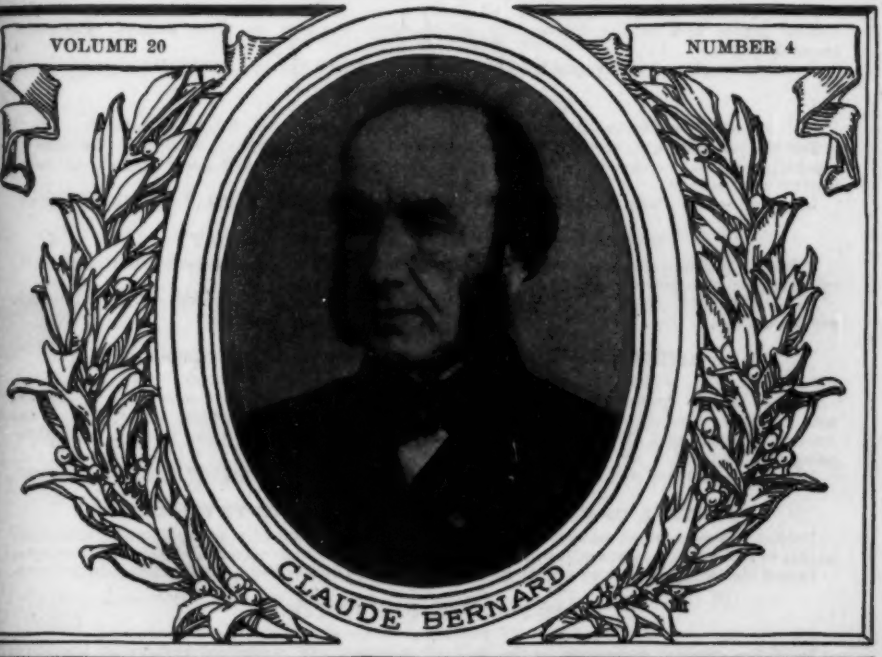
OCTOBER 10, 1940

THE JOURNAL OF NUTRITION

VOLUME 20 NUMBER 4



GEORGE R. COWGILL, *Editor*
Yale University School of Medicine, New Haven, Conn.

WILLIAM H. CHAMBERS, *Assistant to the Editor*
Cornell University Medical College, New York City

ASSOCIATE EDITORS

JOHN B. BROWN — ERNEST B. FORBES — HENRY A. MATTILL
L. H. NEWBURGH — PHILIP A. SHAFFER — HAROLD H. MITCHELL
ALBERT G. HOGAN — ICIE MACY-HOOBLER — LELA E. BOOHER
HOWARD B. LEWIS — ALFRED T. SHOHL

Official Organ of the American Institute of Nutrition
PUBLISHED MONTHLY BY
THE WISTAR INSTITUTE OF ANATOMY AND BIOLOGY
PHILADELPHIA, PA.
Price, $5.00 per volume, Domestic; $5.50 per volume, Foreign; Two volumes issued annually

Entered as second-class matter January 20, 1934, at the post office at Philadelphia, Pa., under Act of March 3, 1879. Copyright 1940, The Wistar Institute of Anatomy and Biology. All rights reserved.

Publications of The Wistar Institute

JOURNAL OF MORPHOLOGY

Devoted to the publication of original research on animal morphology and physiology, including cytology, protozoology, and the embryology of vertebrates and invertebrates. Articles do not usually exceed 50 pages in length.

Issued bimonthly; 2 vols. annually; $20.00 Domestic, $21.00 Foreign, $23.00 Canadian per year.

THE JOURNAL OF COMPARATIVE NEUROLOGY

Publishes the result of original investigations on the comparative anatomy and physiology of the nervous system.

Issued bimonthly; 2 vols. annually; $15.00 Domestic, $16.00 Foreign, $17.50 Canadian per year.

THE AMERICAN JOURNAL OF ANATOMY

Devoted primarily to the interests of anatomy as taught in the medical schools of the United States and to the development of scientific anatomy in these schools. Publishes researches only.

Issued bimonthly; 2 vols. annually; $15.00 Domestic, $16.00 Foreign, $17.50 Canadian per year.

THE ANATOMICAL RECORD

Organ of the American Association of Anatomists and the American Society of Zoologists

For the prompt publication of concise original articles on vertebrate anatomy, preliminary reports; technical notes; critical notes of interest to anatomists and short reviews of noteworthy publications.

Issued monthly; 3 vols. annually; $22.50 Domestic, $24.00 Foreign, $26.25 Canadian per year.

THE JOURNAL OF EXPERIMENTAL ZOOLOGY

Publishes papers embodying the results of original researches of an experimental or analytical nature in the field of zoology.

Issued 9 times a year; 3 vols. annually; $22.50 Domestic, $24.00 Foreign, $26.25 Canadian per year.

AMERICAN JOURNAL OF PHYSICAL ANTHROPOLOGY

Organ of the American Association of Physical Anthropologists

In this journal appear original papers on anthropology in general; researches in evolution, man's origin; human ontogeny; variations in man; heredity; eugenics; demography; the American Indian and abnormal classes. Papers on anthropometry and the methods of anthropology also appear.

Issued quarterly; 1 vol. annually; $6.00 Domestic, $6.50 Foreign, $7.10 Canadian per year.

JOURNAL OF CELLULAR AND COMPARATIVE PHYSIOLOGY

Publishes papers which embody the results of original research of a quantitative or analytical nature in general and comparative physiology, including both their physical and chemical aspects.

Issued bimonthly; 2 vols. annually; $10.00 Domestic, $11.00 Foreign, $12.00 Canadian per year.

THE JOURNAL OF NUTRITION

Organ of the American Institute of Nutrition

Publishes original researches in the field of nutrition and occasional reviews of literature on topics with which the journal is concerned.

Issued monthly; 2 vols. annually; $10.00 Domestic, $11.00 Foreign, $12.00 Canadian per year.

THE AMERICAN ANATOMICAL MEMOIRS

Publishes original monographs based on experimental or descriptive investigations in the field of anatomy which are too extensive to appear in the current periodicals. Each number contains only one monograph. List of monographs already published, with prices, sent on application.

ADVANCE ABSTRACT CARD SERVICE

Every paper accepted for publication in one of the above periodicals is accompanied by the author's abstract. The abstract and the complete bibliographic reference to the paper as it will eventually appear is printed on the face of a standard library catalogue card. This Advance Abstract Card Service is issued promptly, in advance of the journal containing the paper, and is offered in three styles.

Prices per year $2.00, $2.50 and $3.00, postpaid.

These publications enjoy the largest circulation of any similar journals published.

THE WISTAR INSTITUTE OF ANATOMY AND BIOLOGY

Woodland Avenue and Thirty-sixth Street, Philadelphia, Pa.

THE INTRAVENOUS ADMINISTRATION OF CRYSTALLINE AMINO ACIDS TO INFANTS [1]

ALFRED T. SHOHL AND KENNETH D. BLACKFAN

Department of Pediatrics, Harvard Medical School, and the Infants' and the Children's Hospitals, Boston, Massachusetts

(Received for publication May 18, 1940)

That crystalline amino acids may serve as the source of nitrogen for adequate nutrition of laboratory animals has been adequately proved by the long series of experiments initiated by Osborne and Mendel and brought to a successful conclusion by Rose and his collaborators. As far as we know such studies have never been extended to determine the adequacy of pure amino acids for human beings. The preparation of material for this type of experiment is costly and laborious; however, it is only by the use of such purified chemicals that the role of the amino acids in nutrition was determined. When native protein or protein degradation products are used, one is always confronted with the problem of whether the effects obtained are due to the amino acids or to some additional substance in the preparation used. Such was the case when Osborne and Mendel prepared diets from purified amino acids and found it necessary to include "protein-free milk", which carried not only essential minerals and vitamins, but also a small amount of nitrogen of undetermined origin.

Successful nutrition of both infants (Shohl et al., '39) and rats (Mueller et al., '40) has been reported, when the source of nitrogen in the diet consisted of hydrolyzed casein. That this product was free of native protein was attested by the fact that it was found suitable for intravenous use by a number of

[1] This study was supported in part by a grant from Mead Johnson & Company.

THE JOURNAL OF NUTRITION, VOL. 20, NO. 4
OCTOBER, 1940
Copyright 1940.
The Wistar Institute of Anatomy and Biology.
All rights reserved.

investigators. Hydrochloric acid digests of casein are deficient in tryptophane and such preparations must be supplemented with this amino acid before positive nitrogen balances can be obtained (Elman, '39). An enzymatic hydrolysate was found to be a satisfactory source of nitrogen, since positive nitrogen balances were obtained when it was given intravenously with glucose and sodium chloride for short periods of time (Shohl et al., '39). The question of whether positive nitrogen balances in infants could be obtained as well by the use of crystalline amino acids became a matter of great interest.

It was reported that in a few instances of infants given intravenous injections of casein hydrolysate, the only untoward symptom observed was an increase in body temperature. The amount of amino acid administered was large, 2 to 3 gm./lb. of body weight per day (4 to 6 gm./kg.). Quantities of this order are required to obtain adequate positive balances; smaller amounts do not cause a rise in temperature. The question arose as to whether the hydrolyzed casein might contain a pyrogenic substance which could be eliminated during the preparation of the material or removed subsequently. If, on the other hand the property was inherent in the amino acids themselves as thus administered, then the increase in temperature was unavoidable. The most direct method of approach to this problem seemed to be to administer intravenously a mixture containing only crystalline amino acids.

The purpose of our investigation was to compare the effects of crystalline amino acids given intravenously with those of an enzymatic hydrolysate of casein, with regard to temperature reactions and nitrogen balance.

PREPARATION OF MATERIAL

The mixture of amino acids and the casein hydrolysate were supplied to us through the kindness of Dr. W. M. Cox, Jr.[2] The enzymatic hydrolysate of casein contains 11.9% of nitrogen, of which over 60% is present as amino nitrogen. The crystalline amino acid preparation was a modifica-

[2] Of Mead Johnson and Company.

tion of mixture XII of McCoy, Meyer and Rose ('35). The composition is given in table 1. It was necessary to reduce the amounts of tyrosine and cystine used because of their low solubilities. According to the findings of Rose ('38) cystine and

TABLE 1

Amino acid mixtures

	MIXTURE XII [1]	MIXTURE USED FOR INTRAVENOUS INJECTION
	gm.	*gm.*
glycine [2]	3.00	3.00
dl-alanine [2]	3.80	3.80
dl-valine [2]	16.00	16.00
l-leucine [2]	9.00	9.00
dl-isoleucine [2]	8.00	8.00
dl-norleucine [2]	2.50	2.50
l-proline [2]	8.00	8.00
l-hydroxyproline [2]	2.00	2.00
dl-phenylalanine [2]	7.80	7.80
d-glutamic acid [2]	22.00	22.00
d-aspartic acid [2]	4.10	4.10
dl-serine [3]	3.00	3.00
l-tyrosine [3]	6.50	2.00
l-cystine [3]	1.25	0.50
l-histidine HCl [3]	3.40	3.40
d-arginine HCl [3]	6.35	6.35
d-lysine diHCl [3]	11.55	11.55
l-tryptophane [3]	2.25	2.25
dl-methionine [2]	3.50	3.50
d-threonine [4]	—	2.50
$NaHCO_3$	12.86	12.86
	136.86	134.31

[1] McCoy, Meyer and Rose (J. Biol. Chem., vol. 112, p. 283, 1935–1936).
[2] Purchased from Eastman Kodak Company.
[3] Made and supplied by Dr. K. S. Kemmerer, Mead Johnson and Company.
[4] Purchased from Paul Lewis Laboratories, Milwaukee.

tyrosine are not essential amino acids, and the small amount of cystine is compensated in any event by the amount of methionine present. Rose found that threonine was a dietary essential for growth and it has therefore been added. When only the dl form of any acid was available, Rose used twice the amount

of the active form required, even though this might not have been necessary. The presence of hydrochloride in the histidine, arginine and lysine preparations required the inclusion of slightly larger amounts of these materials, in order to supply the requisite amount of amino acid. Rose calculated that 1.424 gm. of his mixture XII are equivalent to 1 gm. of "effective" amino acids. To obtain, therefore, "effective" amino acids equivalent to those of casein or the casein hydrolysate, it would be necessary to use about one-third more of the crystalline preparation. The pH of a 5% solution is 4.4 One hundred grams of the material were available.

The amino acid mixture was prepared for intravenous administration as follows. The dry mixture was dissolved in freshly distilled warm water. With the crystalline acids considerable frothing due to the evolution of CO_2 took place. NaCl and glucose were added, the solution made up to volume and filtered through an autoclaved Berkfeld filter. The water used was obtained the day the solution was to be given, from the general supply of the Hospital which was used for making all the solutions for intravenous use. Since these solutions did not give temperature reactions, they served as a control for the water used. The solutions were used the same day they were prepared. Culture showed them to be uniformly sterile. Because the sample of glucose used was apparently not completely free of nitrogen, the solution was analyzed for nitrogen after it was made.

The amounts of amino acids given as shown in table 2 are the net weights of the crystalline amino acid mixture (minus the $NaHCO_3$) and the nitrogen values are those found by analysis of the injected solution.

PROCEDURE

Preliminary studies were made of the effect on infants of intravenous administration of small amounts of the crystalline amino acid mixture. Observations were made upon six infants who were considered to be normal for the purposes of this study. The solution, prepared as above, contained 5% amino

TABLE 2

*Nitrogen metabolism**

INFANT	NO.	DATE	DURATION		NUTRIMENT						NITROGEN					
			Period	Injection	Type	Volume	Amino Acids		Glucose	Calories	Intake		Urine	Feces	Balance	
			hrs.	*hrs.*		*cc.*	*gm.*	*%*	*%*	*per kg. per 24 hrs.*	*gm.*	*gm./kg./ 24 hrs.*	*gm./ period*	*gm./ period*	*gm./12 hrs.*	*gm./24 hrs.*
	1	11/22/39	24	6	hydrolysate	300	15.0	5.0	5.0	20	1.75	0.30	1.95	0.15		− 0.31
F.	2	12/6–7/39	24	7	hydrolysate	335	16.7	5.0	5.0	23	2.00	0.34	2.24	0.41		− 0.65
age	3	12/8–9/39	24	9	hydrolysate	500	25.0	5.0	5.0	33	2.90	0.49	2.70	0.54		− 0.34
3 mos.	4	12/12–13/39	24	14	crystalline	500	25.0	5.0	5.0	33	2.92	0.49	2.98	0.34		− 0.40
wt.		*1st 12 hrs.*		*12*		*425*					*2.49*		*1.40*	*0.17* [2]	*+ 0.93*	*+ 1.86* [1]
5.9 kg.		*2nd 12 hrs.*		*2*		*75*					*0.43*		*1.58*	*0.17* [2]	*− 1.32*	*− 2.64* [1]
H.	1	12/19–20/39	24	14	hydrolysate	745	25.0	3.3	6.7	65	2.98	0.66	2.78	0.27		− 0.07
age	2	12/27–28/39	24	15	crystalline	840	22.0	2.5	10.0	75	2.46	0.55	2.17	0.06		+ 0.23
2 mos.		*1st 12 hrs.*		*12*		*655*					*1.80*		*1.37*	*0.06*	*+ 0.37*	*+ 0.74* [1]
wt.		*2nd 12 hrs.*		*3*		*185*					*0.66*		*0.80*	*0*	*− 0.14*	*− 0.28* [1]
4.5 kg.	3	12/28–29/39	24		formula [3]	800				120	3.00	0.67	1.95	0.38		+ 0.67
	1	1/12–13/40	24	18	hydrolysate	920	23.0	2.5	7.5	80	2.80	0.60	2.45	0		+ 0.35
		1st 12 hrs.		*12*		*440*					*1.20*		*1.09*	*0*	*+ 0.11*	*+ 0.22* [1]
		2nd 12 hrs.		*6*		*450*					*1.60*		*1.36*	*0*	*+ 0.24*	*+ 0.48* [1]
D.	2	1/13–14/40	24		formula [3]	750					2.40	0.51	1.56	0.38		+ 0.46
age	3	1/16/40	12	12	crystalline	825	21.6	2.5	7.5	70 } 133	2.42	0.52 } 0.83	1.40	0.06	+ 0.96	+ 1.92 [1]
3 mos.	4	1/16–17/40	12		formula [4]	450				63 }	1.44	0.31 }	0.95	0	+ 0.49	+ 0.98 [1]
wt.	5	1/17/40	12		formula [4]	450				126 [1]	1.44	0.62 [1]	0.68	0.16	+ 0.60	+ 1.20 [1]
4.7–	6	1/22/40	12	12	NaCl +											
5.0 kg.					glucose	855	0		10.0	146 [1]	0		0.64	0.07	− 0.71	− 1.42 [1]
	7	1/22–23/40	12		formula [5]	300				84 [1]	0.96	0.41 [1]	0.32	0.15	+ 0.49	+ 0.98 [1]
	8	2/28/40	12	12	NaCl	510	0			0	0		0.88	0.13	− 1.01	− 2.02 [1]
	9	2/28–29/40	12		formula [5]	300				100 [1]	1.45	0.58 [1]	0.64	0.11	+ 0.70	+ 1.40 [1]

* The figures in italics represent subdivisions of the period, the figures for which are directly above.

[1] Calculated for 24 hours.

[2] The value for the two stools was divided arbitrarily.

[3] Five feedings in 24 hours.

[4] Three feedings in 12 hours.

[5] Two feedings in 12 hours.

acids, 5% glucose and 0.45% NaCl. It was given intravenously into a scalp vein, at a rate not usually exceeding 50 cc./hr.[3] At first only 5 cc. of this solution were given. As no febrile reactions or other untoward effects were observed, the amount was increased to 10 cc., then to 25, 50, 100 and 200 cc. Observations of rectal temperatures were made every 30 minutes. When given in these amounts, in no instance was there observed either a rise in temperature or other clinical deviation from normal, regardless of whether the solution was prepared as above or was sterilized by being autoclaved at 20 pounds pressure for 20 minutes before being filtered. We were then ready to increase the amount stepwise, to the point where all the nitrogen and caloric requirements of the infant could be met by material given intravenously.

When amounts greater than 15 gm. (300 cc. of 5% solution) of either the crystalline or hydrolyzed amino acid preparation were given to small infants, in the majority of cases a febrile reaction was observed. So far as we could determine, the temperature increases resulting from the crystalline acid mixture and the hydrolyzed casein mixture were alike. Therefore it was concluded that the effect was due to the amino acids themselves and not to an impurity or hydrolytic by-product in the casein hydrolysate. Further investigation of this problem will be reported elsewhere (Higgins, Shohl and Blackfan, '40).

Three normal white male infants were admitted to the hospital for the purpose of studying nitrogen metabolism. Parallel studies were made with casein hydrolysate and crystalline amino acid solutions on each infant so that the effects of the two materials could be compared. The amount of crystalline amino acid mixture was too limited to permit preliminary observations or lengthy collection periods. The infants were under observation on a constant diet of evaporated milk and corn syrup [4] with supplements of vitamins A, C and D for at least a week before the study was begun. During this fore-

[3] We are indebted to the House Staff of the Infants' Hospital, especially Dr. H. Shwachman for all the intravenous administrations.

[4] Karo.

period they gained in weight, satisfactorily. The general plan in studying the nitrogen metabolism was as follows. The last feeding of milk formula was given at 5 A. M. The infant was placed upon a metabolism bed, the intravenous administration started at about 9 to 10 A. M., and collections of excreta begun. The feces were marked with carmine. The urine was collected for separate 12-hour periods, and preserved with thymol chloroform. Analyses were made for volume, pH, NH_4, urea, amino acids, creatinine and total N of the urine, and of total N of the feces, according to the methods previously described (Shohl et al., '39). [5]

The fractions of N excreted in the urine are not detailed here because they so closely resembled the nitrogen distribution recorded in our former report (Shohl et al., '39). Compared with the findings after milk formula feeding, the amount of urinary amino acids was doubled and undetermined nitrogen was increased at the expense of urea. Examination of the urine showed absence of glucose or at most a trace. The feces following intravenous administration of amino acids were frequently lacking or were so small in amount that the nitrogen content was insignificant.

It was planned to give the solutions over periods of 24 hours, but this could not be carried out for two reasons: first, the flow could not be maintained at a uniform rate and, second, owing to the limitation of the total volume of fluid we were willing to give in a 24-hour period, the administration was necessarily completed at varying times short of 24 hours.

RESULTS

The observations on nitrogen intakes, outputs and balances are given in table 2.

The experiments were conducted consecutively, and the findings in each case led to modifications in subsequent procedure. Therefore the three cases are detailed separately.

In periods F1 and F2 the intakes of both nitrogen and calories were low. In F3 and F4 the nitrogen intake was

[5] We are indebted to Wilma Stanley Hill for most of the analyses and to Harriet O'Hara for the care of the infants in the Metabolism Ward.

higher, but the caloric intake only slightly increased. Under these conditions nitrogen equilibrium was not attained. The results obtained by equal amounts of the casein hydrolysate and the crystalline amino acid mixture (F3 and F4) were indistinguishable.

Realizing that the second 12-hour period was practically a starvation period, the urine for the two 12-hour periods was analyzed separately in F4. This clearly differentiates the first 12-hour period, which showed a considerable positive balance, from the second 12 hours which showed a considerable negative balance.

In the second infant, H., the caloric intake was augmented by increasing both the amount and the concentration of glucose in the solution. Although the amino acid concentration was lower, the total nitrogen per kilogram of body weight was higher than in F. Under these conditions, nitrogen equilibrium was attained for the whole 24-hour period with the casein hydrolysate (H1). Positive nitrogen balance was attained with crystalline amino acids (H2) although the nitrogen intake was lower than in H1, presumably because the caloric intake was significantly higher. Again as in F4, separation of the nitrogen output into two 12-hour periods shows a positive balance for the first 12 hours and negative balance for the second 12 hours, during 9 hours of which the infant fasted.

In the third infant, D, with a smaller amount of casein hydrolysate (D1) than in H1, and slightly increased caloric intake, positive balance for the 24 hours was attained. When this experiment is separated into 12-hour periods, it is shown that each short period gave a positive balance. In this experiment approximately equal amounts of nitrogen were given in the two 12-hour periods, although the rate was increased in the second 12-hour period. The 6-hour fast did not cause a negative nitrogen balance.

The crystalline amino acids were given (D3) more rapidly than in F4 and H2. The administration was completed in 12 hours. The nitrogen balance was positive and high. To obviate the effects of starvation, the infant was fed milk formula

during the second 12-hour period (D4) thus increasing both nitrogen and caloric intakes for the 24-hour period. This second 12-hour period also showed a positive nitrogen balance, as did a subsequent 12-hour formula period (D5).

When saline and glucose solution was given (D6) without amino acids, under exactly comparable conditions, nitrogen was lost at the rate of 1.4 gm. per day. When saline only (D8) was given, the rate of nitrogen loss was 2.0 gm. per day.

Observations were made on a period of milk formula feeding immediately following each amino acid administration after the period H2. The purpose was to determine whether the material given in intravenous administration and recorded as positive balance was subsequently excreted. If such had been the case, negative balances would have occurred in cases H3, D2 and D4. These periods, however, show positive balances of the order expected from the intakes. This is particularly well shown in D4 and D5, which not only show a normal percentage retention of nitrogen, but closely resemble each other.

The periods D7 and D9, following nitrogen starvation, demonstrate the avidity with which the body retains nitrogen to compensate for previous losses. In these two cases the amount of nitrogen excreted in the urine was actually smaller than when no nitrogen was given and the percentage of the nitrogen retained was raised to nearly half the amount consumed.

From these data it is concluded that crystalline amino acids and casein hydrolysate administered intravenously were equally well utilized by the body as shown by the fact that each resulted in retention of nitrogen, and that the retained nitrogen was not lost in the after-periods.

DISCUSSION

When the crystalline amino acid mixture and the casein hydrolysate were compared, approximately equal amounts of nitrogen were administered, although nearly one-fourth of the amino acids of the crystalline product were in the inactive form. It has been customary in nutrition experiments, when racemic amino acids are fed, to increase the amount of amino

acids to make up for the inactive material present. The inclusion of increased amounts of inactive amino acids reduces the proportion of some of the essential acids in the crystalline mixture. Therefore, when this mixture is used at low levels of intake, inadequacy of certain essential acids may result. At higher levels of intake the absolute amounts are sufficient regardless of proportion. However, some of the forms not occurring naturally in protein are apparently just as useful as the natural form, for growth. We desired to test the crystalline amino acid mixture under rigorous conditions, and therefore used amounts corresponding to the casein hydrolysate instead of larger amounts. It is of considerable interest, therefore, that the nitrogen balances are quite comparable for the two mixtures of amino acids. The nitrogen retentions with the casein hydrolysate and the crystalline mixture were so similar in parallel observations that they cannot be differentiated in these experiments.

Short periods of observation of nitrogen balance are valid only if the subject has attained a steady state under the conditions to be studied. The nitrogen retentions do not have quantitative validity unless the conditions have been constant for 5 to 7 days. If one goes from a high level of intake to a lower but still adequate one, there will be a transition period with negative balances or low retentions until positive balances are again established on the lower intake. Conversely when the intake is increased, temporary high positive balances result until the conditions again become stable. The fore-periods included ingestion of greater amounts of nitrogen (3.5 to 4.8 gm. N/day) than were given intravenously, so that the tests were made under conditions which were not favorable to positive balances.

The results with saline and glucose alone (D6) are a measure of the negative nitrogen balances which would have occurred had the injected nitrogen not been utilized by the body. These values could be used to correct the nitrogen balances obtained for the periods of fasting. No such calculations have been made, however, because qualitatively the meaning of the bal-

ances is clear without such computation, and the quantitative values have not sufficient validity to justify it. They do demonstrate, however, in a striking manner that the amino acids were utilized by the body and that they converted what would otherwise have been considerable nitrogen loss to large positive balances.

When food is given by mouth it is absorbed slowly and hence consecutive 12-hour periods do not show alternating plus and minus nitrogen balances. Amino acids administered intravenously must be utilized when presented and therefore their metabolism is more rapid than that of a corresponding amount of material given by mouth. This was demonstrated by the great variation in nitrogen excretion in successive 12-hour periods.

It may be argued that part of the amino acids given in any period was excreted in the following 12 hours. This would result in either a diminished positive balance or a negative balance in the second 12-hour period, as was the case in F4 and H2. This argument, however, does not take account of two other important factors, namely, the total calories given in 24 hours and the duration of fasting. When there is a long fast, (H2), nitrogen catabolism continues (as in D8) resulting in negative nitrogen balance. This rate of excretion is increased when fasting follows a period in which inadequate calories were given (F4). The conditions were most severe in F4 when a fast of 10 hours followed a period of inadequate caloric intake, less severe in H2 when more calories were supplied in the first 12 hours. When sufficient nourishment was given in the second 12-hour period even with a 6-hour fast (D1) negative balance was prevented. The most favorable results are found in D3 and D4, in which a high positive balance during intravenous administration was followed by positive balance in the second 12 hours, during which there was adequate caloric intake and no period of fasting.

Therefore, the negative balances in the second 12-hour period of F4 and H2 do not demonstrate delayed excretion and hence lack of utilization of ingested nitrogen, but taken in

conjunction with periods D1, D2, D3, D4, D6 and D8 do allow us to evaluate the excretion on the basis of the several factors involved. The conclusion is reached that for successful intravenous nutrition not only must adequate nitrogen and calories be supplied, but the administration should be continuous or at least without long periods of fasting.

The present studies confirm and extend our previous observations that, in conjunction with adequate fluid, glucose and sodium chloride, 0.5 gm. of N/kg./day given intravenously to a normal infant, will result in satisfactory nitrogen retentions for short periods of time.

SUMMARY

The rise in temperature which sometimes accompanies the intravenous administration of amino acids was found to be caused equally by casein hydrolysate and a crystalline amino acid mixture.

Positive nitrogen balances of the same degree were obtained by intravenous administration of similar amounts of a crystalline amino acid mixture and casein hydrolysate.

LITERATURE CITED

ELMAN, R. 1939 Time factor in retention of nitrogen after intravenous injection of a mixture of amino acids. Proc. Soc. Exp. Biol. & Med., vol. 40, pp. 484–487.

HIGGINS, H., A. T. SHOHL, AND K. D. BLACKFAN 1940 Unpublished data.

MCCOY, R. H., C. E. MEYER, AND W. C. ROSE 1935 Feeding experiments with mixtures of highly purified amino acids. VIII. J. Biol. Chem., vol. 112, pp. 283–302.

MUELLER, A. J., K. S. KEMMERER, W. M. COX, JR., AND S. T. BARNES 1940 The effect of casein and a casein digest on growth and serum protein regeneration. J. Biol. Chem., vol. 134, p. 573.

ROSE, W. C. 1938 The nutritive significance of the amino acids. Physiol. Rev., vol. 18, pp. 109–136.

SHOHL, A. T., A. M. BUTLER, K. D. BLACKFAN, AND E. MACLACHLAN 1939 Nitrogen metabolism during oral and parenteral administration of the amino acids of hydrolysed casein. J. Pediat., vol. 15, pp. 469–475.

FAT AS A FACTOR IN THE HEALING OF RICKETS WITH VITAMIN D[1, 2]

ARTHUR KNUDSON AND ROBERT J. FLOODY
Department of Biochemistry, Albany Medical College, Albany, New York

ONE FIGURE

(Received for publication, March 7, 1940)

The study of the effects of various factors on the efficiency of vitamin D action is very important. In the course of some studies in this laboratory, a diet of purified foodstuffs, low in lipids, was devised. This diet was modified so that it could be used for studies in experimental rickets, and a study of the influence of fat on the healing of rickets by vitamin D was undertaken.

McDougall ('38) reports that rats eating a diet containing a high proportion of wheat flour or bread, and low in calcium and vitamin D, were prevented from developing rickets by addition of 11% lard or olive oil. She ascribes these results to the fact that absorption of calcium is promoted by formation of calcium soaps, and in absence of fat, calcium combines with phytin (or its cleavage product) to form an insoluble compound which cannot be absorbed. Recent experiments of Boer ('39) have likewise indicated that rats on a rachitogenic diet are prevented from developing rickets when 10% margarine is added. This effect is ascribed to the fatty acids having an antirachitic action in the presence of small amounts of vitamin D; in other words, fats have a sparing action on vitamin D. Kon and Booth ('34 a, '34 b) are of a similar opinion.

[1] Presented before the American Chemical Society, Milwaukee, Wisconsin, September, 1938.

[2] This work was aided by a grant from the Winthrop Chemical Company.

EXPERIMENTAL

Young piebald rats, weighing from 45 to 60 gm., were used as experimental animals and were placed on the rachitogenic diet at about 25 days of age. At the end of the 21 days, when a uniform degree of rickets had developed as evidenced by x-ray pictures, the animals were then ready for the test period. As a basis of procedure for determining the efficiency of vitamin D, we followed essentially the method for assay of vitamin D in cod liver oil, as described in the U. S. Pharmacopeia XI. The test period was 10 days, and on the eleventh day, examination for the degree of healing was judged by both the x-ray and the line test. The degree of healing by the line test was indicated by the following conventional rating used in this laboratory (Knudson and Benford, '38): 1+ indicates a broken line of calcified bone; 2+, a narrow continuous line; 3+, a broad continuous line; 4+, practically complete calcification.

The percentage composition of the rachitogenic diet (6C) is as follows: lactalbumin 7HAAX [3] 18, sucrose 72, salt mixture 2, rice polish concentrate [3] 5, calcium carbonate 3. To each kilogram of this food mixture are added 2 mg. of riboflavin dissolved in alcohol, and 20 mg. of carotene dissolved in ether. The calcium and phosphorus contents were 1.27 and 0.25%, respectively; the Ca to P ratio was 5 to 1.

The salt mixture used in the above diet is a modification of the Osborne and Mendel salt mixture no. XXX ('26–'27), free from calcium and phosphorus, and used by them in rachitogenic diets. It is simplified from the Osborne and Mendel salt mixture in that only solid ingredients are used. Its composition (in grams) is as follows: magnesium citrate 31.0, magnesium sulfate 11.3, potassium chloride 128.0, sodium chloride 33.2, ferric citrate ($1\frac{1}{2}$ H_2O) 6.34, manganese sulfate ($2H_2O$) 0.02, sodium fluoride 0.248, and potassium aluminium sulfate ($K_2Al_2(SO_4)_4$, 24 H_2O) 0.0538.

The lactalbumin, 7HAAX, is a highly purified preparation, free from lipid material, calcium, and phosphorus. Rice polish

[3] Obtained from The Borden Company, New York.

concentrate contains from 0.4 to 0.9% lipid material, and 4.6% of phosphorus. The phosphorus content of the diet is therefore derived practically entirely from the rice polish. The total lipid content of the diet is less than 0.05% and this is derived from the rice polish concentrate. The calcium and

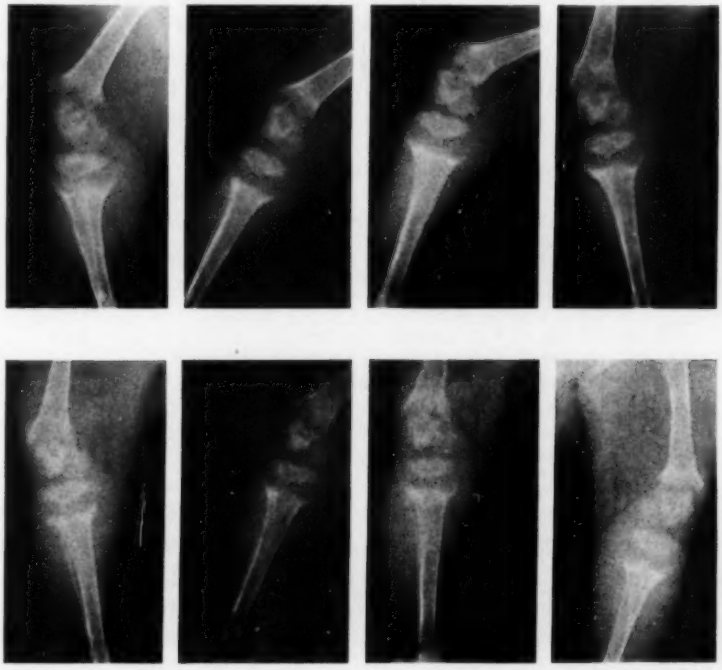


Fig. 1 The upper row shows the x-ray photographs of knee points of rats at the end of 3 weeks' subsistence on the 6C diet. The lower row shows the knee joints of rats at the end of 3 weeks on the Steenbock-Black 2965 diet.

phosphorus contents of the above diet are about the same as those for the Steenbock-Black diet 2965 ('25). The only unpurified material in diet 6C is the rice polish concentrate.

A marked uniform degree of rickets is produced with this ration, which is similar to the degree of rickets obtained with the Steenbock-Black 2965 diet. In figure 1 are presented x-ray

photographs of knee joints of rats showing the development of rickets on these two diets at the end of 3 weeks.

The ash contents of fat-free, dry extracted femurs of rats on the 6C diet, and Steenbock-Black diet, for 21 days, are also very similar. The average of ten rats on the 6C diet showed an ash content of 28.9%, which may be compared with 29.9% for the Steenbock-Black 2965 diet. The healing effects produced by vitamin D when given to rats on these two diets also show excellent agreement. There were twenty rats on each test dose with each of the diets and the results are given in table 1.

TABLE 1

Comparison of healing of rickets in rats subsisting on 6C diet and Steenbock-Black diet 2965

DATE	MATERIAL TESTED	DEGREE OF HEALING	
		6C diet	Steenbock-Black diet 2965
2–12–38	35 mg. U. S. P. reference cod liver oil	3.1+	2.8+
2–21–38	35 mg. U. S. P. reference cod liver oil	2.9+	2.7+
2–10–38	0.08 μg. vitamin D_2 in propylene glycol	2.5+	2.5+
2–12–38	0.10 μg. vitamin D_2 in propylene glycol	3.2+	3.1+
2–18–38	0.08 μg. vitamin D_2 in propylene glycol	3.1+	3.4+
2–21–38	0.10 μg. vitamin D_2 in propylene glycol	3.5+	3.3+

To study the effect of fat on the healing of rickets by vitamin D, 5, 10 or 20% of a hydrogenated fat [4] was substituted for a corresponding amount of sucrose in the 6C diet. For comparison, similar amounts of fat were substituted for gluten flour in the Steenbock-Black 2965 diet. The results of this study are presented in table 2.

In the first series of experiments, 0.025 μg. of crystalline vitamin D_2 [5] dissolved in propylene glycol, was added to 40 gm. of diet. This amount of diet was consumed in all cases within 6 to 7 days. It will be noted that the healing is about three times greater with 5% of fat in the diet than with no fat. With 10% of fat, the healing is markedly lessened, and with 20%

[4] Crisco.

[5] We are grateful to Dr. O. W. Barlow of the Winthrop Chemical Company for supplying the crystalline vitamin D_2.

of fat, even more so. The Steenbock-Black 2965 diet itself contains 2.75% of total lipids, so that in these experiments slightly more fat was present than in those on the 6C diet. In the second series of experiments, the same amount of vitamin D_2 was used except that it was given by mouth in four doses on alternate days, i.e., on the first, third, fifth, and seventh

TABLE 2

Effect of fat on the healing of rickets

FAT IN DIET	6C DIET		STEENBOCK-BLACK 2965 DIET	
	Number of rats	Healing	Number of rats	Healing
Series I. 0.025 μg. vitamin D_2 in propylene glycol added to 40 gm. diet				
No fat added	10	0.6+	10	0.6+
5%	10	2.2+	10	1.7+
10%	10	1.6+	10	0.9+
20%	10	1.2+	10	0.7+
Series II. 0.025 μg. vitamin D_2 in propylene glycol given separately in four doses on alternate days (1, 3, 5, 7 day)				
No fat	10	0.9+	10	0.8+
5%	10	2.0+	10	1.1+
10%	10	1.7+	10	0.8+
20%	10	1.3+	..	....
Series III. 0.1 μg. vitamin D_2 dissolved in propylene glycol given separately in four doses on alternate days (1, 3, 5, 7 day)				
No fat	20	1.45+	20	1.4+
3%	10	1.55+	..	....
5%	20	2.25+	20	2.3+
10%	20	1.2 +	20	1.8+

days. While the results are not quite so striking, nevertheless, they do indicate that 5% of fat added to the diet is more effective than 10 or 20% in bringing about healing of rickets. In the third series of experiments 0.1 μg. of vitamin D_2 was given separately in four doses by mouth and a similar result was obtained with 5% of fat added to the diet. In one of the experiments in this series on the 6C diet, only 3% of fat was present and the healing was practically no greater than with no fat.

In all of these experiments, whether vitamin D was given by mouth or separately, 5% of fat in the diet brought about much greater healing than with no fat. Larger amounts of fat in the diet, such as 10 or 20%, produced progressively less healing, although the healing was generally greater than with no fat.

DISCUSSION

The fact that 5% of fat in the rickets diet produces a much better healing with a known amount of vitamin D than does a diet with 10 or 20% of fat, is very significant. One may well ask how this smaller amount of fat produces its effect, and why a larger amount of fat should be less effective. Ten and 20% of fat in the diet are by no means to be considered excessive. McDougall ('38) expressed the view that the rachitogenic action of cereals is due to a deficiency of fat, and that a normal amount of fat seems to be important for the solubility in the intestine of calcium and phosphorus. She points out that the fatty acids produced by hydrolysis form calcium soaps which are then rendered soluble by the action of bile and thus readily absorbed. That the formation of calcium soaps in the presence of fat does have some influence on the healing of rickets obtained with vitamin D seems evident from a series of experiments in which the calcium carbonate in the 6C diet was replaced by an equivalent amount of calcium in the form of calcium oleate, or calcium stearate. The fatty acid combined with calcium in this diet was about equivalent to the total fatty acids that would be derived from the hydrolysis of 20% of fat in the diet. In another series of experiments, one-fourth of the calcium carbonate was replaced by an equivalent amount of calcium in the form of an oleate or stearate. This would be equivalent to the fatty acids obtained with 5% of fat in the diet. The results of these tests are given in table 3.

It is to be noted that when all of the calcium is present in the form of calcium oleate or stearate or an equivalent mixture of both, there is considerably more healing than with calcium in the form of a carbonate. There is, however, no significant

difference between healing obtained with the calcium in the form of an oleate or a stearate. When only one-fourth of the calcium carbonate is replaced by an equivalent amount of calcium in the form of an oleate or stearate, the healing is about the same as with calcium carbonate. These results are extremely interesting, and rather surprising. In the series with calcium soaps containing combined fatty acids equivalent to the fatty acids obtained from the hydrolysis of 5% of fat

TABLE 3

Effect on the healing of rickets by vitamin D of replacement in the 6C diet of calcium in the form of a carbonate by calcium in form of a soap

FORM OF CALCIUM IN DIET	NUMBER OF RATS	DEGREE OF HEALING
0.025 μg. vitamin D given separately in 4 doses		
Control on 6C diet	10	1.2+
¼ $CaCO_3$ replaced by an equivalent amount of Ca in the form of stearate	10	1.5+
All $CaCO_3$ replaced by an equivalent amount of Ca in the form of stearate	10	2.3+
¼ $CaCO_3$ replaced by an equivalent amount of Ca in the form of oleate	10	1.4+
All $CaCO_3$ replaced by an equivalent amount of Ca in the form of oleate	10	2.0+
¼ $CaCO_3$ replaced by an equivalent amount of Ca in the form of a mixture of equal parts oleate and stearate	10	1.2+
All $CaCO_3$ replaced by an equivalent amount of Ca in the form of a mixture of equal parts oleate and stearate	10	2.1+

in the diet, there is no more healing than with the fat-free controls, which is in marked contrast to the greatly increased healing obtained with 5% of fat in the diet. However, in the series with the calcium soaps containing combined fatty acids equivalent to the fatty acids obtained from 20% of fat in the diet, the healing is much greater than with 20% of fat in the diet. The results of these tests with calcium soaps would seem to contradict those given in table 2 with added amounts of fat to the diet. In view of these apparently contradictory

results it seems evident that the greater effectiveness of 5% of fat in the diet over 20% of fat in the diet cannot be explained on the basis of the formation of calcium soaps.

Boyd, Crum and Lyman ('32) have indicated that the presence of a moderate amount of fat in the diet may have a beneficial influence on absorption of calcium and phosphorus by maintaining a favorable acidity of the intestinal contents. On the other hand, Nicolaysen ('38) did not obtain any greater utilization of calcium on a diet with 20% of fat than when no fat was added to a fat-poor ration consisting of corn, wheat-gluten, sodium chloride, and calcium carbonate. Zucker and Barnett ('23) explain the influence of fat on the prevention of rickets as due to the formation of insoluble calcium soaps, and phosphate in soluble form which would otherwise be precipitated as calcium phosphate, is made available for absorption.

These various viewpoints do not offer us an explanation for the fact that 5% fat is more effective than 10 or 20% of fat in the healing of rickets with vitamin D. It may be possible that more than one factor is concerned with this interesting phenomenon and that before any explanation can be given, more information than is covered in our experiments or those of others, will have to be obtained.

SUMMARY

1. A rachitogenic diet has been devised which is made up largely of purified materials and which is low in lipids.

2. Addition of 5% of fat to the rachitogenic diet gave a much better healing with a known amount of vitamin D than with no fat in the diet.

3. With the addition of 10 or 20% of fat, the healing of rickets by vitamin D was less than with 5% of fat, but greater than was obtained with no fat.

4. Replacement of all the calcium carbonate in the 6C diet by an equivalent amount of calcium in the form of an oleate or stearate, resulted in better healing. Replacement of only

one-fourth of the calcium carbonate by an equivalent amount of calcium in the form of an oleate or stearate did not result in any appreciable difference in the healing with vitamin D.

LITERATURE CITED

BOER, J. 1939 The influence of the fat content of the diet on the production of rachitis in rats. Acta Brevia Neerland. Physiol. Pharmacol. Microbiol., vol. 9, p. 67.

BOYD, OSCAR F., CARLOS L. CRUM AND J. F. LYMAN 1932 The absorption of calcium soaps and the relation of dietary fat to calcium utilization in the white rat. J. Biol. Chem., vol. 94, p. 29.

KNUDSON, A., AND F. BENFORD 1938 Quantitative studies of the effectiveness of ultraviolet radiation of various wavelengths in rickets. J. Biol. Chem., vol. 124, p. 287.

KON, STANISLAW KAZIMIERZ, AND ROLAND GORDON BOOTH 1934 a The vitamin D activity of butter. II. The complex nature of the antirachitic action of butter. Biochem. J., vol. 28, p. 111.

———— 1934 b The vitamin D activity of butter. III. An attempt to elucidate the nature of the labile factor in butter antirachitic for the rat. The antirachitic potency of lard, olive oil, egg oil and the fatty acids of butters and lard. Biochem. J., vol. 28, p. 121.

MCDOUGALL, E. J. 1938 The counteraction by fat of the anticalcifying action of cereals. Biochem. J., vol. 32, pp. 194–202.

NICOLAYSEN, R. 1938 Fat and calcium metabolism. Tids. Kjemi Bergvesen, vol. 18, p. 141–143.

OSBORNE, T. B., AND L. B. MENDEL 1926–1927 Nutrition. Carnegie Institution Year Book, no. 26, p. 351–358.

STEENBOCK, H., AND A. BLACK 1925 The induction of growth-promoting and calcifying properties in fats and their unsaponifiable constituents by exposure to light. J. Biol. Chem., vol. 64, p. 263.

ZUCKER, T. F., AND MARION BARNETT 1923 Observations on the distribution of anti-rachitic substances. Proc. Soc. Exp. Biol. Med., vol. 20, p. 375.

THE DETERMINATION OF ASCORBIC ACID IN EVAPORATED MILK, POWDERED MILK AND POWDERED MILK PRODUCTS [1]

WARREN W. WOESSNER,[2] C. A. ELVEHJEM AND HENRY SCHUETTE

Department of Chemistry, College of Letters and Science, and Department of Biochemistry, College of Agriculture, University of Wisconsin, Madison

(Received for publication May 18, 1940)

Within recent years evaporated and powdered milks have received some consideration as a potential source of vitamin C. This problem has apparently received little attention in America.

Schlemmer, Bleyer and Cahnmann ('32), Henriksen ('37), Palladina and Anoshkina ('37), Taniguti, Hamamoto, Hirata and Suzuki ('37), Tomoi and Tomita ('37), Henry and Kon ('38), Kon ('38) and Henry, Houston, Kon and Osborne ('39) have studied the ascorbic acid content of different milk preparations. Phospho-18-tungstic acid and various modifications of the indophenol titrimetric technique were used by them. In most cases the work on the milk preparations themselves was not verified by biological assay nor was any evidence presented as to the specificity of the reaction involved in the analysis.

Further study of the photoelectric method suggested by Woessner, Elvehjem and Schuette ('39) has disclosed certain improvements especially valuable when determining dehydroascorbic acid. We wish therefore to describe the improved method as applied to evaporated milk, powdered milk, and

[1] Published with the approval of the Director of the Wisconsin Agricultural Experiment Station.

[2] University Fellow, Department of Chemistry; present address, E. I. duPont and Company, New Brunswick, New Jersey.

powdered milk products and to cite experimental evidence that the method is indeed specific for ascorbic acid.

EXPERIMENTAL PART

The samples of evaporated milk were prepared by diluting one part of the evaporated milk with one part of copper-free water. Samples of powdered milks and powdered milk products were prepared by dissolving 145 gm. of the powder in 1 liter of copper-free water. Heating to 50°C. was necessary in order to bring the roller-dried products into solution. All powders were stirred into the water with an ordinary silver-plated spoon.

The samples were analyzed for both the reduced and reversibly oxidized ascorbic acid. The reduced ascorbic acid was determined according to the procedure already described by the authors ('39). However a very slow sliding of the galvanometer is always observed with evaporated milk. Thus it is necessary to take the galvanometer reading not later than 15 seconds after the indophenol has been added to the colorimeter tube if the true reduced ascorbic acid is to be determined accurately.

The procedure for the determination of the dehydroascorbic acid has been modified as follows: After the addition of a few drops of dibutyl phthalate to the milk in order to prevent foaming, wet hydrogen sulfide is bubbled through the milk for exactly 20 minutes. Then as rapidly as manipulation will permit, 25 ml. of the hydrogen-sulfide-saturated milk are added to 75 ml. of modified Willberg reagent and the whole shaken well to break the resulting curd into small particles. The hydrogen sulfide is removed immediately by passing a vigorous stream of wet oxygen-free nitrogen through the suspension. Our experience has shown that this operation takes no longer than 20 minutes for 50 ml. of the curd-reagent mixture. After the curd is removed by filtration, 5 ml. of the filtrate are measured into one of the colorimeter tubes. We have never experienced difficulty in obtaining a crystal-clear serum at this point. Then with the simultaneous start of a stop watch

10 ml. of the dye-acetate solution are added to the tube by means of a rapidly delivering pipette and the galvanometer readings at 15 and 30 seconds are recorded. The galvanometer reading corresponding to zero seconds, for practical purposes, can be considered equal to the difference of the galvanometer readings at 15 and 30 seconds subtracted from the galvanometer reading at 15 seconds.

The calculations and other details of this modified procedure are identical with those described in the original paper. This modified procedure is also recommended for use with whole milks, raw and pasteurized, as it gives more reliable results especially when determining dehydroascorbic acid.

DISCUSSION

Experiments conducted with dehydroascorbic acid solutions in 10% aqueous sodium chloride prepared either by iodine oxidation or use of ascorbic acid oxidase of Tauber, Kleiner and Mishkind ('35) showed that at hydrogen ion concentrations comparable to those of fresh milk and milk preparations recovery of dehydroascorbic acid was quantitatively complete when hydrogen sulfide was introduced for 20 minutes or longer. It was not possible to obtain 100% recovery at a pH 2 or lower even after 8 hours of hydrogen sulfide treatment. This is in accordance with the experiences of Bessey ('38) but not in agreement with the observation of Kon and Watson ('36).

Like de Haas and Meulemans ('36), we have observed that hydrogen sulfide treatment of milk reduces other substances besides dehydroascorbic acid. Since these reduced substances react with the indophenol at a slower rate than ascorbic acid at the final pH used in the colorimeter tube, the interference can be eliminated by extrapolation to zero time. However if the hydrogen sulfide treatment is extended much beyond 20 minutes, it becomes increasingly difficult to extrapolate with acceptable accuracy.

Heated lactose solutions (pH 6) introduce no error. However when heated lactose solutions were treated with hydrogen

sulfide under the conditions of the experiment a very slow movement of the galvanometer was observed. The interference was not serious and could be eliminated by extrapolation to zero time.

Redissolved washed casein did not contribute to the formation of interfering substances upon hydrogen sulfide treatment. It was found that at the pH of milk riboflavin offered no interference as it did with the original method at the higher hydrogen ion concentrations. Hydrogen sulfide treatment of lactoglobulin solutions prepared from evaporated milks produced a marked interference very similar to that observed under the conditions of the procedure suggested. This interference also could be eliminated by extrapolation to zero time. The lactalbumin fraction of milk also indicated formation of interfering substances on treatment with hydrogen sulfide but to a much less marked degree. This interference was likewise successfully eliminated by extrapolation.

The fact that these effects were more marked with the more drastically heated milk products than with the raw or pasteurized milks, seems to indicate that reducible soluble proteins or protein fractions have been formed during the processing. It is very likely also that some of these reducible substances are sulfhydryl compounds. Josephson and Doan ('39) reported that sulfhydryl compounds are formed in milk if it is heated to a sufficiently high temperature. Their observation that these compounds are readily oxidized with copper is in agreement with the observations made in this laboratory. They suggest that the lactalbumin of the milk is the most likely ingredient responsible for this condition and state that the protein of the fat globule adsorption membrane may also be involved.

Since qualitative examination of a white flocculent precipitate obtained from a strongly ammoniacal evaporated milk serum gave a positive test for tin and a very faint test for iron, the reaction of these metals in the procedure was studied. Stannic and stannous tin solutions did not accelerate the oxidation of ascorbic acid under semi-anaerobic conditions.

Stannic tin itself does not interfere although when treated with hydrogen sulfide it contributed to the formation of stannous tin which partially remains in solution under the conditions of the method. At the final pH in the colorimeter tube (4.1) the stannous tin reacts at a slower rate than the ascorbic acid. Hence no error is introduced if the readings are extrapolated to zero time.

The case of iron is more serious. Ferric iron does not react with 2,6-dichlorophenolindophenol but the hydrogen sulfide treatment always produces some ferrous iron which remains in solution. The ferrous iron reacts instantaneously with the indophenol at the final pH of the solution in the colorimeter tube. Since milk picks up, at most, an insignificant quantity of iron from the processing which it undergoes, the error introduced from this source under ordinary conditions is nil. A sample of evaporated milk, by analysis found to contain a total of 15 mg. of ascorbic acid per liter, was treated for 15 minutes with the ascorbic acid oxidase of Tauber, Kleiner and Mishkind ('35). Analysis then showed no reduced ascorbic acid present but treatment with hydrogen sulfide for 20 minutes indicated 15 mg. of dehydroascorbic acid per liter. If the oxidase-treated milk was heated to 100°C. for 10 minutes, the analysis showed no ascorbic acid. However if either copper, hydrogen peroxide or intense irradiation was used to destroy completely the ascorbic acid present, analyses would always indicate reducing activity following hydrogen sulfide treatment. Since no reducing activity was ever obtained after hydrogen sulfide treatment, when use was made of ascorbic acid oxidase followed by heat, it is deemed that a non-specific oxidizing procedure, besides causing the irreversible destruction of ascorbic acid, will also cause the formation of substances which, when reduced with hydrogen sulfide, are capable of instantaneous reaction with indophenol at pH 4.1. If iron had been present in sufficient quantity to interfere, the oxidase-treated heated sample would have given a positive value for dehydroascorbic acid. Furthermore, tests on the serum of the milk before and after hydrogen sulfide treatment

failed to give a positive reaction for iron. The value of the ascorbic acid oxidase in deciding whether or not all reducing activity is due to ascorbic acid is only valid if interference due to stannous tin and reduced proteins can be eliminated, for we have observed that ferrous iron and stannous tin are oxidized by ascorbic acid oxidase. Since copper also catalyzes these reactions, this may be evidence in support of the essential constituent of the enzyme.

Further assurance that the method is specific for ascorbic acid was given by biological assay of samples of evaporated milk which showed wide variance in their ascorbic acid content. The same samples showed similar potency by biological and chemical assay.

Results of the determinations by method presented. The results compiled in tables 1 and 2 were secured in the normal process of testing the method under working conditions. Since it was not possible to obtain information as to the age of the samples, the time of the year they were prepared or other pertinent data, they are included here to give an indication of the ascorbic acid content of powdered and evaporated milks as purchased throughout bakery, grocery, and drug stores in a city of moderate size. A few general conclusions may also be drawn from the data.

It is apparent from tables 1 and 2 that the ascorbic acid content of powdered milk is roughly equal to that of evaporated milks. The powdered milk products, however (items 8 and 9 in table 1 for example), are for the most part significantly inferior to powdered milks and the average evaporated milk. It appeared that neither the spray process, the roller process, nor even the mode of storage, is the significant factor which determines the ascorbic acid content of the powder. It is possible that, unless special precautions are used, a greater quantity of heat-labile dehydroascorbic acid is formed the more the powdered milk and powdered milk products are handled or modified. Thus, when the dehydration is brought about by heat, the destruction of the antiscorbutic potency is in direct proportion to the quantity of dehydroascorbic acid

TABLE 1

Ascorbic acid content of powdered milks and powdered milk products

MATERIAL TESTED	ASCORBIC ACID PER LITER[1]			
	Reduced	Oxidized	Total	Per kg. of powder
	mg.	mg.	mg.	mg.
1. Open barrel, skim, spray	9.0	2.4	11.5	78.8
2. Open barrel, skim, spray	1.3	1.2	2.6	17.9
3. Closed tin, skim, roller	7.2	1.8	9.1	62.7
4. Open barrel, whole, roller	9.6	4.4	14.0	97.1
5. Vacuum, whole, spray	9.4	1.2	10.6	68.6
6. Spray dried, modified by addition of milk fat and sugar. Vacuum	7.9	0.4	8.3	57.9
7. Superior quality milk, part of fat removed, fortified by rice extract; irradiated and dried by roller process, vacuum	13.4	1.2	14.6	100.8
8. Protein milk, high in protein, lactic acid and low in lactose, pH 4.8, vacuum	2.6	4.3	6.9	48.0
9. Soluble casein, lactalbumin, lactose, milk salts, dextrins, maltose, extracts of yeast and wheat embryo. Vacuum	1.8	0.0	1.8	12.6

[1] Powder was dissolved to give a milk containing 12.5% of total solids.

TABLE 2

Ascorbic acid content of commercial evaporated milk

PRODUCT TESTED	ASCORBIC ACID PER LITER[1]		
	Reduced	Oxidized	Total
	mg.	mg.	mg.
Brand 1	3.0	0.8	3.8
Brand 2	3.9	1.5	5.4
Brand 1	4.6	2.4	7.0
Brand 3	4.9	4.7	9.6
Brand 4	8.0	1.6	9.6
Brand 5	11.2	1.5	12.7
Brand 6	11.8	1.9	13.7
Brand 7	10.0	4.1	14.1
Brand 8 Irradiated	19.5	2.8	22.3
Brand 8 Irradiated	20.1	0.3	20.4
Brand 9 Zuker Process	13.7	5.7	19.5
Brand 10 Irradiated	7.8	0.1	7.9
Brand 11 Irradiated	1.7	5.7	7.4
Brand 10 Irradiated	6.5	0.2	6.7
Evaporated goats' milk	14.8	0.0	14.8

[1] As milk comes from can, undiluted. Divide by two in order to convert to milligrams per liter of normal whole milk.

present. Consequently it appears that powdered milks are potentially a significant source of antiscorbutic factor if they are prepared from a milk which is rich in reduced ascorbic acid at the time of manufacture.

We have observed that milk can be aerated at 100°C. for 5 minutes with small loss of ascorbic acid. Consequently it is not surprising to find that a milk powder dissolved at 20°C. or at 50°C. will yield milks which show no detectable difference in their ascorbic acid content if the milk prepared at the higher temperature is cooled soon after its preparation.

The data of table 2 reveal that the ascorbic acid content of irradiated evaporated milks is not significantly different from that of the non-irradiated. This is understandable in view of the observation made by Scheer ('39) that only a slight reduction in ascorbic acid occurs when milk is irradiated with the intent of fortifying it with vitamin D and that this reduction is without practical significance.

The Evaporated Milk Association cooperated with this investigation in the collection of twenty-one samples of evaporated milk manufactured during the first week of April, 1940, in various plants throughout this country. The analyses of these samples are given in table 3. The sterilization temperature was 242° F. for 14 minutes and the forewarming or preheating temperatures varied from 204° F. to 212° F. Analyses were completed 12 to 16 days after the samples were manufactured.

The data reveal that the metallic nature of the manufacturing equipment is not the limiting factor which determines the ascorbic acid content of evaporated milk. This is proved by the wide variance in ascorbic acid shown by milks manufactured in equipment consisting of various combinations of copper, tinned copper and stainless steel. Evaporated milks manufactured almost entirely in copper equipment may contain more ascorbic acid than some evaporated milks manufactured almost entirely in stainless steel equipment.

The survival of any ascorbic acid at all while the milk is being subjected to evaporation in a copper pan may be ex-

plained by the presence of sulfides which are formed when the milk is heated, as well as a scarcity of oxygen. The sulfides possibly combine with any trace of copper which may dissolve and also act as reducing agents during the process. The latter point is not entirely a speculation in the light of observations by Sharp, Trout and Guthrie ('36) and Josephson and Doan ('39) who showed that ascorbic acid is protected to a considerable degree when the milk is heated to sufficiently high temperatures even when small amounts of copper are present. Josephson and Doan ('39) postulate that this action

TABLE 3

Ascorbic acid content of evaporated milks[1]

LOCATION OF PLANT	MATERIAL OF CONSTRUCTION[2]			ASCORBIC ACID PER LITER[3]		
	Pre-heater	"Hot wells"	Evaporating pan	Reduced	Oxidized	Total
				mg.	*mg.*	*mg.*
Colorado	none	Sn-Cu	Cu	24.0	3.6	27.6
Idaho	none	Ni	Cu	13.8	2.0	15.8
Illinois	Cu	S.S.	Cu	13.3	2.5	15.8
Indiana	S.S.	S.S., Cu	S.S.	15.8	0.0	15.8
Iowa	S.S.	Sn-Cu	Cu	12.0	0.0	12.0
Kentucky	Sn-Cu	Ni	Cu	7.2	0.8	8.0
Maryland	S.S.	Cu	S.S.	5.0	1.2	6.2
Michigan	S.S.	S.S., Cu	S.S.	6.2	1.8	8.0
Minnesota	Cu	Sn-Cu	Sn-Cu, S.S.	22.4	1.6	24.0
Mississippi	none	Ni	Cu	7.2	2.8	10.0
Missouri	S.S.	S.S. or Cu-Ni alloy	S.S.	11.6	2.2	13.8
Ohio	S.S.	Sn-Cu	Cu	11.6	2.2	13.8
Oregon	S.S.	Sn-Cu, S.S.	Cu	10.0	3.8	13.8
Tennessee	S.S.	Ni	Cu	5.0	3.0	8.0
Texas	Sn-Cu	Sn-Cu	Cu	4.6	2.6	7.2
Utah	none	Ni	Cu	20.8	0.0	20.8
Virginia	S.S.	S.S.	Cu	6.7	3.3	10.0
Washington	S.S.	Sn-Cu	Cu	4.0	0.0	4.0
Wisconsin	S.S.	S.S.	Cu	17.6	1.6	19.2
Wisconsin	none	Cu	Cu	14.8	1.0	15.8
Wisconsin	Cu	glass	Cu	7.7	0.8	8.5
			Average	11.5	1.7	13.2

[1] Supplied by the Evaporated Milk Association.

[2] Sn-Cu, tinned copper; S.S., stainless steel.

[3] As milk comes from the can, undiluted. Divide by two in order to convert to milligrams per liter of normal whole milk.

is due to the formation of sulfhydryl compounds in the milk at the elevated temperatures.

We have observed that its dehydroascorbic acid is completely destroyed when whole milk, containing about equal quantities of ascorbic and dehydroascorbic acids, is heated for only 10 minutes at 100°C. Thus it is understandable that the dehydroascorbic acid content of evaporated milk is insignificant and for practical purposes its determination could be omitted. The very small quantity of dehydroascorbic acid found indicates that this acid is completely destroyed during the evaporation and sterilization of the milk. Apparently sulfhydryl compounds or sulfides cannot inhibit the thermal rupture of dehydroascorbic acid. Consequently the actual quantity of ascorbic acid which survives during the processing is largely dependent on the quantity of the acid in the reduced form in the milk as it is received before concentration.

In attempting to prevent ascorbic acid losses it is apparent, from table 3, that either the omission or inclusion of a preheating step in the process of manufacture is not important. Likewise neither the quality nor the quantity of the stabilizing salts used exerts any influence here. Moreover the ascorbic acid content cannot be correlated with geographical site of production of the evaporated milk.

If the values cited for commercial evaporated milks in tables 2 and 3 are divided by two in order to express them on the basis of whole milk, we find that the average American evaporated milk supplies 25% of the quantity of ascorbic acid found in freshly drawn cow's milk. It should be emphasized, also, that this observation is based upon the analyses of evaporated milk samples which were fresh, that is, stored for no longer than 2 weeks. That a different picture may be obtained after the evaporated milks have been stored for a longer period, is quite probable inasmuch as Henry, Houston, Kon and Osborne ('39) observed that ascorbic acid is lost slowly but steadily under such conditions. We have a range, however, of 1.9 to 13.8 mg. of ascorbic acid per liter. This range indicates that evaporated milk is potentially a signifi-

cant source of ascorbic acid. Raw milk contains on an average 22.6 mg. of ascorbic acid per liter. This demonstrates that in certain of the samples of evaporated milk only about a 50% loss of ascorbic potency has occurred. The maximum value of 13.8 mg. per liter is not much lower than the average value of 17.3 mg. per liter, which we have reported for commercial raw milk, nor is it significantly different from the average value of 12.6 mg. per liter which we have reported for commercial pasteurized milk.

SUMMARY

The improvements of the method previously suggested by the authors make the method less time-consuming and the determination of dehydroascorbic acid more reliable. Among the substances found to make the use of the photoelectric colorimeter essential are heated lactose, stannous tin and certain of the water soluble proteins or protein fractions. The reliability of the photoelectric colorimeter is limited only by iron which fortunately is not present in significant quantities in milk or milk products.

The analyses of forty-one evaporated milks and nine powdered milks are given. The data reveal that the metallic nature of the manufacturing equipment is not the limiting factor which determines the ascorbic acid content of evaporated milk.

LITERATURE CITED

BESSEY, O. A. 1938 A method for the determination of small quantities of ascorbic acid and dehydroascorbic acid in turbid and colored solutions in the presence of reducing substances. J. Biol. Chem., vol. 126, p. 771.

DE HAAS, J. H., AND O. MEULEMANS 1936 The ascorbic acid content of human milk in Batavia (trans. title). Geneeskund. Tijdschr. Nederland-Indie, vol. 76, p. 2277.

HENRIKSEN, P. K. 1937 Vitamins and milk. Ingeniøren, vol. 46, p. 61; 1938, Chem. Zentr., vol. 109, I, p. 1025.

HENRY, K. M., J. HOUSTON, S. K. KON AND L. W. OSBORNE 1939 The effect of commercial drying and evaporation on the nutritive properties of milk. J. Dairy Res., vol. 10, p. 272.

HENRY, K. M., AND S. K. KON 1938 The effect of commercial sterilization on the vitamin C content of milk. J. Dairy Res., vol. 9, p. 185.

JOSEPHSON, D. V., AND F. J. DOAN 1939 Cooked flavor in milk. Milk Dealer, vol. 29, no. 2, p. 35.

KON, S. K. 1938 The effect of commercial sterilization on nutritive values of milk. VII. Conclusions. J. Dairy Res., vol. 9, p. 207.

KON, S. K., AND M. B. WATSON 1936 The effect of light on the vitamin C of milk. Biochem. J., vol. 30, p. 2273.

PALLADINA, O. K., AND A. A. ANOSHKINA 1937 The vitamin C content of milk and its synthesis during lactic fermentation. Microbiology (U.S.S.R.), vol. 6, p. 787; 1938, Chem. Zentr., vol. 109, I, p. 459.

SHARP, P. F., G. M. TROUT AND E. S. GUTHRIE 1936 Vitamin C, copper and the oxidized flavor of milk. Tenth Ann. Report. N. Y. State Assoc. of Dairy and Milk Inspectors, p. 153.

SCHEER, K 1939 Schädigt die Ultraviolettbestrahlung der Milch ihren Vitamin-C-Gehalt? Münch. Med. Wochschr., vol. 86, p. 603.

SCHLEMMER, F., B. BLEYER AND H. CAHNMANN 1932 Studien über Biochemishe Aktivatoren der Milch. Biochem. Z., vol. 254, p. 187.

TANIGUTI, T., E. HAMAMOTO, Y. HIRATA AND K. SUZUKI 1937 Kritik über die Vitamin-C-bestimmung nach indophenoltitration. Orient. J. Diseases Infants, vol. 21, p. 1.

TAUBER, H., I. S. KLEINER AND D. MISHKIND 1935 Ascorbic acid (vitamin C) oxidase. J. Biol. Chem., vol. 110, p. 211.

TOMOI, T., AND S. TOMITA 1937 Vitamin C contents of different milk preparations. Sei-i-kai Med. J., vol. 56, p. 469; C. A., vol. 31, p. 8725[3].

WOESSNER, W. W., C. A. ELVEHJEM AND H. A. SCHUETTE 1939 The determination of ascorbic acid in commercial milks. J. Nutrition, vol. 18, p. 619.

A MODIFICATION OF THE LINE TEST APPLICABLE TO CHICKEN VITAMIN D ASSAY

E. W. McCHESNEY AND E. HOMBURGER

Biological Laboratories, Winthrop Chemical Company, Inc., Rensselaer, New York

FOUR TEXT FIGURES AND TWO PLATES (SIX FIGURES)

(Received for publication June 24, 1940)

Remp and Marshall ('38), in a report on the antirachitic activity of various forms of vitamin D in the chick, made use of an "index of calcification" devised by one of us (E.H.) and obtained an excellent correlation between this index and the per cent of bone ash. They did not, however, describe the manner in which the index was determined. It is our purpose, in this paper, to describe the manner in which a rapid index of the degree of calcification in chick bones may be determined, by a modification of the U.S.P.XI line test, and to present data on the correlation of this index with the bone ash and the dosage of vitamin D. Although we have, in this laboratory, studied more than 700 chicks by the methods to be described below, the present report deals only with 240 birds which were put on test specifically for this paper.

OUTLINE OF TEST METHOD

The test animals were male white Leghorn chicks which were received in the laboratory in December, 1938, on the second day of life. They were separated into fourteen groups of twelve to eighteen birds each. The experimental procedures followed were those described by Remp and Marshall. The chicks were fed the modified diet of Hart, Kline and Keenan ('31), which was supplemented by 2% of corn oil for the negative control group, and 2% of cod liver oil (assaying

100 U.S.P. units of vitamin D per gram) for the positive control group. However, the chicks which received vitamin D supplements were medicated daily by direct (stomach tube) injection into the crop with the following exceptions: During the first 4 days (second to sixth day of life) none of the birds were given supplements in order to avoid excessive handling, but from the seventh to tenth days of life the daily vitamin dosage was doubled, thus compensating for the lack of initial treatment. After the tenth day of life the several groups of birds received the regular daily dosage at the levels indicated in the table. Body weights were recorded weekly, and the chicks were sacrificed on the thirtieth day of life. The two tibiae were removed, cleansed of soft tissues, and fixed in 90% ethyl alcohol for at least 48 hours, or until convenient for examination.

For our final selection of animals for inclusion in this report, we have used certain standards of growth. Only those birds have been included which gained at least 250% of their initial weight. We consider birds falling below this standard as too stunted to be representative. This criterion of selection leaves at least half the birds in each group, and in most of the groups leaves over 75%. From the selected chicks, one tibia was taken for the determination of bone ash as directed in the method of the Association of Official Agricultural Chemists (Lapp, '39), and the other tibia reserved for sectioning and staining as described below. The two assay procedures were carried out independently by the two authors, neither having knowledge of the results obtained by the other until the data were complete.

DESCRIPTION OF THE VITAMIN PREPARATIONS TESTED

The crystalline vitamin D_3 tested was prepared by the method of Windaus, Lettre and Schenck ('35). This compound has a formula of $C_{27}H_{44}O$, and a melting point of 82–84°C. The maximum absorption was at 265 mμ, the specific rotation in acetone was $[\alpha]_D^{20} = +83°$. The vitamin D_3-cholesterol corresponded in all respects to crystalline vitamin

D_3 with the exception that purification was less complete, so that the antirachitic potency was only 20,000 international units per milligram instead of 40,000 international units per milligram as in the case of the crystallized principle. The irradiated cholesterol in oil (Delsterol) was purchased on the open market in sufficient quantities of one lot for all tests. The antirachitic potency of all the preparations was determined initially by the standard U.S.P. rat method, using the U.S.P. Reference Oil and the International Standard for vitamin D.

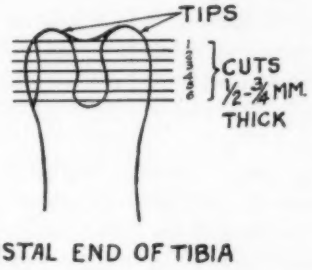


Fig. 1 Illustration of method of sectioning chicken bones

METHOD FOR THE DETERMINATION OF THE INDEX OF CALCIFICATION

The distal end of one of the paired tibiae from each chick is taken for the purpose of sectioning. The bone is cut with a razor blade, or other suitable instrument, at right angles to the long axis of the bone. After the condyles have been cut off—and a point is reached at which each cut includes an entire cross section of the bone—six or seven serial cuts of about 0.4 to 0.6 mm. in thickness are made, as illustrated by figure 1. This is as thin a cut as one can usually make by hand with a razor blade. The sections are immersed at once in 2% silver nitrate, and arranged serially; a shallow dish or watch glass may be used for this purpose. When the sections become suitably dark (time depending on the amount of light, but usually in 5 to 10 minutes), they are ready for evaluation. For this purpose a binocular microscope permitting 2 or 3

magnifications is desirable. Except for the method of making the cuts and the serial arrangement, the procedures differ in no important respect from the standard line test.

In order to evaluate the results, all of the sections are taken into consideration. If examination reveals that a questionable or borderline deviation from normal has occurred, the sections may be turned over and another reading taken. The principal points to be studied are: (1) size of the calcified areas, (2) density of calcification within these areas, whether solid or spotty (but in this connection one must not confuse unevenness of staining), and (3) shape of the calcified areas, whether well-formed or irregular in outline. A definitely normal bone, comparable to the positive controls, is recorded as 3+ (+++) to 4+ (++++). Calcification below this level is recorded as in the schema for the official line test. Negative controls show great variations in the degree of rickets present, but do not give anything comparable to plus readings. They are therefore recorded as (—), or (——) if the rickets is very severe. A number of photographs illustrating several of the degrees of response are shown in the accompanying plates (figs. 2 to 7 inclusive). The amount of magnification used for each photograph was 2 times.

RESULTS

One tibia from each chick was used for visual examination as described above. The reliability of the modified line test as an index of the antirachitic effects of vitamin D was checked by determining the percentage of bone ash on the opposite tibia. These data, together with those on body weights, are presented in table 1.

Responses to the vitamin D_3 dietary supplement were the most typical. There was satisfactory growth at every dose level, and an increase in the average per cent bone ash for each increase in the vitamin supplement until the positive control level was reached. In contrast, the average index of calcification as noted visually continued to increase with each increment of vitamin supplement. The vitamin D_3-cholesterol

gave a satisfactory growth and the index of calcification likewise increased with each increase of vitamin supplement; but, the observed bone ash values are inconsistent since the average value at the 2-unit dose level exceeded those noted at either the 3-unit or 6-unit level. The reason for this is not clear. The irradiated cholesterol preparation gave a rather poor response in all particulars, especially in growth. Under the conditions of the experiment the positive control group also did not gain weight as rapidly as expected.

TABLE 1

Response of chicks to treatment with vitamin D

GROUP NO.	VITAMIN PREP'N.	DAILY DOSE	BODY WEIGHT		INDEX OF CALCIFICATION, AVERAGE	BONE ASH, AVERAGE
			Original median	Gain, median		
		I.U.[1]	gm.	%		%
1	Vitamin D_2	1	33	366	+1.38 (±0.77)	38.02 (±4.38)
2	"	2	33	410	+2.56 (±0.33)	44.64 (±0.91)
3	"	3	34	405	+3.05 (±0.18)	44.98 (±1.53)
4	"	6	33	461	+3.38 (±0.30)	44.95 (±1.10)
5	D_3 cholesterol	1	32	433	+2.41 (±0.68)	43.40 (±2.02)
6	"	2	32	435	+2.88 (±0.24)	45.61 (±1.24)
7	"	3	32	398	+2.96 (±0.30)	44.03 (±0.86)
8	"	6	34	469	+3.33 (±0.44)	45.11 (±0.87)
9	Irradiated cholesterol	1	34	294	+0.31 (±0.96)	36.29 (±2.68)
10	"	2	33	354	+2.23 (±0.59)	42.02 (±2.35)
11	"	3	34	378	+2.89 (±0.49)	44.03 (±2.05)
12	"	6	36	298	+3.07 (±0.34)	45.64 (±1.02)
13	2% corn oil (diet alone)		36	300	−1.50 (±0.49)	31.57 (±0.76)
14	2% cod liver oil in diet		37	306	+3.34 (±0.31)	44.82 (±0.81)

[1] International units.

The chief purpose of this paper, however, is to emphasize the relationships which we have found to exist between the per cent of bone ash and our index of calcification, and of these factors in turn to the vitamin dosage. In order to bring out these relationships more clearly, they are presented in graphic form in figures 8, 9 and 10.

Figure 8 illustrates the observed relationship of dosage of vitamin D to the per cent of bone ash for all three prepara-

tions tested. The curves will be seen to be typical except in the case of the vitamin D_3-cholesterol, as mentioned above. It would be very difficult to decide from the bone ashes alone whether the prophylactic dose lies between 1 and 2 units or between 3 and 6 units. The irregularity noted might have been eliminated by using a larger number of birds per group than employed in this study, although we used the standard number (fifteen to eighteen).

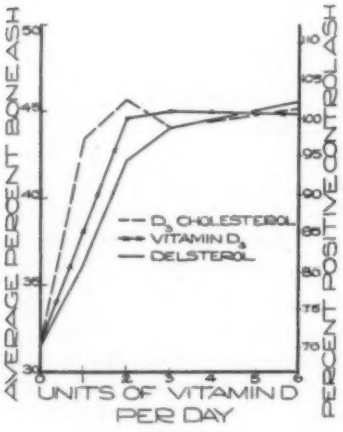


Fig. 8 Relationship of dosage of vitamin D to per cent of bone ash.

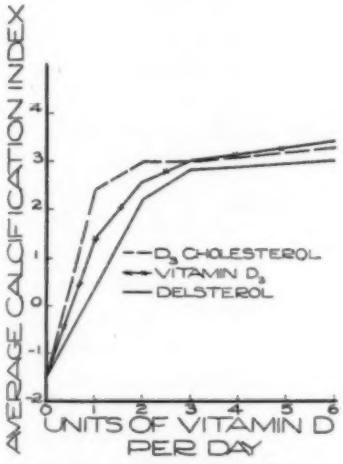


Fig. 9 Relationship of dosage of vitamin D to index of calcification.

Figure 9 illustrates the relationship of dosage of vitamin D to the index of calcification. The curves are much the same in character as those for the bone ash, showing an increase of the index with each increase of the vitamin supplement. One important difference between this factor and the observed ash values is worthy of mention: after a certain degree of calcification occurs, the bone ash may show only insignificant changes, while use of the suggested modification of the line test permits the better bone formation to be demonstrated.

Hence the index continues to rise after the observed bone ash has reached a plateau. We regard an index of calcification of +3 as normal and, with this as a basis, a clear-cut result may be deduced for the potency of each of the three preparations tested.

Figure 10 illustrates the relationship of the per cent of bone ash to the index of calcification as taken from the points given in the table. These points fall approximately on a straight line. However, in the case of individual chicks, there is not

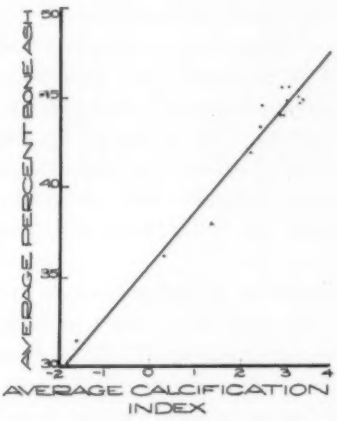


Fig. 10 Relationship of average per cent bone ash to average index of calcification.

always a good correlation between the two criteria, largely we believe because of greater variability in results obtainable by ashing methods. Thus a chick which gives a bone ash of 44% may give an index of +3.5, while we may find in another chick a bone ash of 47% and an index of +3.0. Nevertheless, the two criteria show in general a good correlation in the majority of cases and the mean values by the two methods approximate each other to an increasing degree with doses of 2 or more units daily.

DISCUSSION

Several criteria have thus far been used for vitamin D assay with chickens: (1) per cent of bone ash, (2) width of metaphysis, and (3) the index of calcification. Each of these has its value and all appear to give results which agree rather well with each other. We have not correlated the width of metaphysis with other indices in this report, but it was shown by Remp and Marshall to agree well with the bone ash and index of calcification. Of these three procedures, the bone ash method would ordinarily be the most time-consuming. It should be noted that the time required for the bone ash determination may be considerably reduced by ashing the bones in groups instead of individually, but even then the preliminary preparation involving cleaning, crushing, extracting and drying is a long procedure. The width of the metaphyses may of course be determined in less time than the index of calcification. Our experience indicates that this index will give information qualitatively as reliable as either of the other two criteria.

A further illustration of the extent to which the index of calcification and per cent bone ash are in agreement is seen in the conclusions which we would draw from each regarding the potency of the vitamin preparations tested in this report. If we consider an index of calcification of 3+ and a bone ash of 44.82% to be normal, as indicated by these assays, we arrive at the following conclusions as to the prophylactic doses of the three preparations: By bone ash, vitamin D_3, 2.5 I.U.; D_3-cholesterol, 1.7 (or 5.2) I.U.; irradiated cholesterol, 4.4 I.U. By the visual or line test index of calcification the corresponding doses are: vitamin D_3, 2.90 I.U.; D_3-cholesterol, 3.1 I.U.; Delsterol (irradiated cholesterol), 4.8 I.U.

SUMMARY

1. An adaptation of the U.S.P. line test to chick bones is described. The method permits a rapid and accurate determination of the index of calcification and an excellent corre-

lation between the degree of calcification and dietary supplements of vitamin D.

2. A typical chicken vitamin D assay is given including data on both the index of calcification and the per cent bone ash.

3. It is shown that both criteria would lead to substantially the same result for the potency of the various preparations of vitamin D tested.

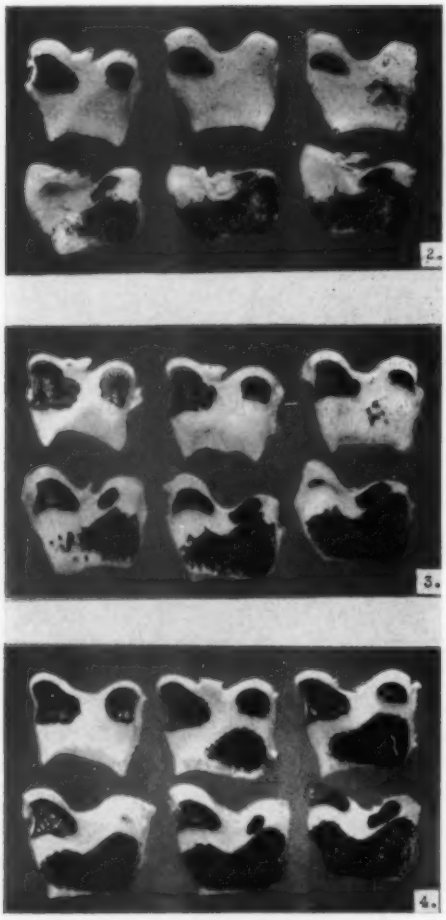
LITERATURE CITED

HART, E. B., O. L. KLINE AND J. A. KEENAN 1931 A ration for the production of rickets in chicks. Science, vol. 73, p. 710.

LAPP, M. E. (Comp.) 1939 Vitamin D assay by preventive biological test. J. Assn. Offic. Agr. Chem., vol. 22, p. 82.

REMP, D. G., AND I. H. MARSHALL 1938 The antirachitic activity of various forms of vitamin D in the chick. J. Nutrition, vol. 15, p. 525.

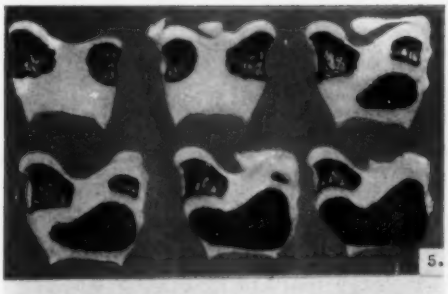
WINDAUS, A., H. LETTRE AND F. SCHENCK 1935 Über das 7-dehydrocholesterin. Ann. Chem., Bd. 520, S. 98.

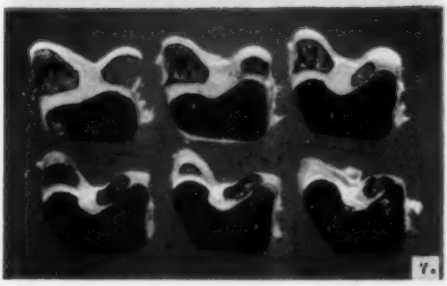


2 Index of calcification, —2; bone ash, 30.71%. Note almost complete absence of diaphyseal calcification in the first three sections, very poor and spotty diaphyseal calcification in the last three sections: poor bone formation.

3 Index of calcification, +0.75; bone ash, 36.67%. Note larger epiphyseal areas of calcification than above, but small diaphyseal areas except in the last section (and even this is not well-formed).

4 Index of calcification, +1.5; bone ash, 40.18%. Note the greater amount of diaphyseal calcification, first beginning to appear in the second section. There are large diaphyseal areas in the last three sections but none are well-formed.





5 Index of calcification, +2; bone ash, 42.80%. Note the diaphyseal areas of calcification do not begin to appear until the third section. However, those areas in the fifth and sixth sections are well-formed.

6 Index of calcification, +3.0; bone ash, 44.90%. Note that there are large diaphyseal areas of calcification in all but the first section. Excellent formation of bone is evident in the last three sections particularly.

7 Index of calcification, +3.5; bone ash, 47.80%. Note excellent bone formation throughout and the large well-formed diaphyseal area of calcification even in the first section. Bone was almost too hard to cut.

THE COMPOSITION OF GAINS MADE BY RATS ON DIETS PROMOTING DIFFERENT RATES OF GAIN [1]

MARJORIE PICKENS, WILLIAM E. ANDERSON AND ARTHUR H. SMITH
Laboratory of Physiological Chemistry, Yale University School of Medicine, New Haven

ONE FIGURE

(Received for publication June 5, 1940)

The rate of gain in body weight of the rat has been widely used in nutrition experiments as an important criterion of the adequacy of nutrition. During the last 20 years, as the increasing knowledge of dietary essentials has been applied to the feeding of laboratory animals, the rate of increase in body weight considered normal for this species has risen strikingly. Probably the most rapid rate produced by exclusively dietary means is that obtained by Anderson and Smith ('32). The present contribution includes an amplification of that work.

Another phase of development that has aroused interest is the ability of rats to grow after a period of stunting. According to Jackson and Stewart ('20), the nature, severity and duration of the deficiency as well as the age of the animal are factors affecting the rate of recovery from stunting. These generalities have been amply confirmed by Clarke and Smith ('38).

The purpose of the present investigation was to determine the composition, in terms of water, lipids, ash and protein, of the material added when rats gain in weight at different rates.

[1] The material for this paper was taken from a dissertation submitted by Marjorie Pickens in partial fulfillment of the requirements for the degree of Doctor of Philosophy, Yale University, 1937.

The five rates represented were: the rapid rate obtained by Anderson and Smith ('32), that accepted as standard for this colony, two rates of retarded growth and an intermediate rate of gain obtained at a mature age during the recovery of the stunted animals.

EXPERIMENTAL

Male albino rats weighing from 38 to 46 gm. at 21 ± 1 days were divided into four experimental groups. For one group, referred to as the rapidly growing animals, the diet of modified calf meal,[2] paste food [3] and liberal vitamin supplements employed by Anderson and Smith ('32) was adopted in order to reproduce the rapid rate of gain observed by them. In addition to these foods, which were provided ad libitum, these animals received 3 gm. of dried yeast twice weekly and 20 gm. of lettuce daily.

For another group, representing a "normal" rate of gain, the stock diet of the colony was employed. Between the twenty-first and forty-second days this regimen was identical with that described above except that only 10 gm. of lettuce was given once weekly. From the forty-second to the forty-ninth day 3.5 gm. of paste food, approximately half the amount previously consumed, was allowed daily. On the forty-ninth day paste food was entirely withdrawn and calf meal remained the principal food. After the forty-second day the animals received 20 gm. of lettuce once weekly, 1 gm. of yeast three times weekly, and 1 gm. of wheat germ three times weekly.

A third group, gliadin-stunted rats, received ad libitum between the twenty-first and one hundred and tenth days a diet

[2] Maynard ('30). The modified calf meal used in this work was made up of 3% of cod liver oil and 97% of a mixture obtained from the Cooperative G. L. F. Exchange, Buffalo, New York, having the following percentage composition: Linseed oil meal 15, corn meal 20.75, ground malted barley 10, wheat red dog flour 22, dried skim milk 12, oat flour 15, soluble blood meal 3, salt 1, steamed bone meal 1, cod liver oil 0.25.

[3] The paste food consisted of 25% of whole milk powder, 25% of commercial casein, 20% of wheat germ and 30% of lard.

consisting of gliadin 18%, dextrin 51%, salt mixture (Osborne and Mendel, '19) 4%, hydrogenated vegetable oil [4] 22% and cod liver oil 5%. At the age of 110 days these animals were realimented by substituting an equivalent amount of lactalbumin for gliadin in the diet. The fourth group, calorie controls for the third, was fed between the forty-second and one hundred and tenth days this lactalbumin diet in the same amounts in which the gliadin-stunted rats voluntarily consumed their gliadin diet. After realimentation at 110 days both groups were fed the lactalbumin diet ad libitum. As a source of the vitamin B complex the stunted animals received throughout the experiment 200 mg. daily of dried yeast. Intended to be an adequate allowance at all ages, it was, in view of later assays [5] of this brand of yeast, probably inadequate in the latter period of the experiment.

Animals of all dietary groups were weighed and the weight of food consumed was noted every 4 days. Approximately eight animals were killed at birth and at 14 and 21 days of age, and eight were killed from each of the four dietary groups at 42, 110 and 230 days of age, and from the two stunted groups at 160 days as well.

The whole bodies freed of the contents of the gastrointestinal tracts were analyzed for water, fat, nitrogen and ash by modifications of methods employed by Light et al. ('34). The ground carcasses together with washings from the chopper were dried in vacuo at 55°C. to constant weight (0.1%). Lipids were determined on the entire ground carcass because it was found that accurate sampling for subsequent determinations was possible only on "dry fat-free matter." The material reported as lipids is that obtained by extracting the "dry matter" with boiling 4:1 mixture of anhydrous alcohol and ether, filtering, evaporating, dissolving the residue in ether, evaporating and drying overnight in a vacuum oven. All desiccations and evaporations were carried out under carbon dioxide and the solvents were freshly distilled in order to prevent oxida-

[4] Crisco.

[5] Private communication from Dr. G. R. Cowgill.

tion. The iodine number of the freshly isolated fat was determined by the Hanus method on a few samples of each diet and age group.

The "dry fat-free matter" was dried overnight in a vacuum oven, weighed and ground to a very fine, light powder in a ball mill or feed mill. Nitrogen was determined on 0.3 gm. samples of this material by the Kjeldahl method using metallic selenium as catalyst. Ash was determined on 1 gm. samples of the same material by incineration in a muffle furnace at 560°C.

RESULTS AND DISCUSSION

Increase in body weight. The growth curves of the four groups are given in figure 1. The rapidly growing animals gained at a slightly lower rate than those of Anderson and Smith ('32), requiring 25 days instead of 23.3 days to increase from 60 to 200 gm. in body weight. The records of the growth of the two stunted groups describe two levels of retarded rate of gain, and, in spite of differences in type of deficiency and in initial rate of growth, they show practically identical rates of recovery from stunting. Although the growth of recovery represents the most rapid growth of the stunted animals, it is distinctly inferior to the early gains of the rapidly growing and stock animals.

Recovery of food energy in the gains. Although this experiment was not designed to study the utilization of food energy, data on food intake were recorded and have been related to amounts of protein and lipids recovered in the gains. The energy content of the foods and of the protein and lipids of the gains was calculated, using the factors 4, 4 and 9 calories per gram for carbohydrate, protein and fat, respectively. For commercial products manufacturers' analyses were employed. As is to be expected, the proportion of food energy accounted for as fat and protein in the gains made by the two rapidly growing groups declined as these animals increased in weight. However, the stunted animals, as is indicated in table 1, showed some improvement in storage of food energy during

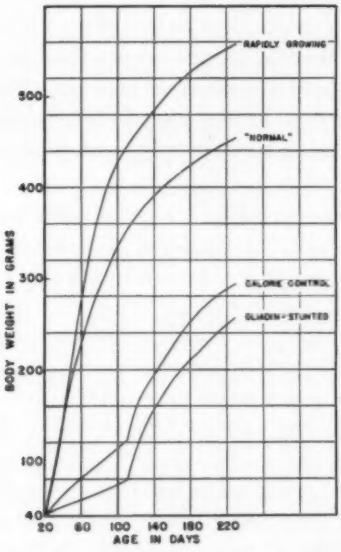


Fig. 1 Average growth curves of experimental groups.

TABLE 1

Recovery of food energy in gains made by stunted animals

EXPERIMENTAL GROUP	PERIOD (DAYS)	ENERGY CONSUMED IN FOODS		ENERGY ACCOUNTED FOR IN GAINS		PERCENTAGE OF FOOD ENERGY ACCOUNTED FOR IN GAINS AS LIPIDS AND PROTEIN
		Calories	Calories per gram of gain	As lipids (calories)	As protein (calories)	
Calorie controls	21– 42	412	18.7	...	18	2.1
	42–110	1169	20.5	54	47	8.6
	110–160	1945	19.0	148	70	11.2
	160–230	3438	50.5	114	56	4.9
Gliadin-stunted	21– 42	412	58.9	1	6	0.4
	42–110	1169	35.4	24	20	3.8
	110–160	1655	15.3	159	81	14.5
	160–230	3059	57.9	70	52	4.0

the period of deficient feeding. This response may imply an adaptation to the deficient diets.

Composition of bodies. The average values for the composition of the bodies of the experimental animals of the various groups are presented in table 2. The data for individual animals have been compared statistically by the method for small groups described by Snedecor ('37). The differences

TABLE 2

Average composition of bodies

EXPERIMENTAL GROUP	AGE	SET WEIGHT	COMPOSITION OF WHOLE BODIES					COMPOSITION OF FAT-FREE MATERIAL		
			Water	Lipids	Protein (N × 6.25)	Ash	Dry fat-free matter	Water	Protein	Ash
	days	*grams*	%	%	%	%	%	%	%	%
	Birth	6.23	86.06	1.78	9.01	1.44	11.72	87.6	9.2	1.5
	14	22.30	73.61	9.36	13.24	2.20	16.79	81.2	14.6	2.4
	21	39.9	67.98	13.20	13.69	2.08	17.42	78.3	15.8	2.4
Rapidly growing	42	161.9	64.4	13.8	16.6	2.2	20.97	74.7	19.3	2.6
	110	445.9	57.0	21.1	16.2	2.6	20.55	72.2	20.5	3.2
	230	540.9	53.4	24.7	15.8	2.7	19.93	70.9	21.5	3.6
Stock	42	147.1	65.9	12.5	16.5	2.5	21.29	75.3	18.9	2.9
	110	350.3	62.0	13.9	19.3	3.2	24.02	72.0	22.4	3.7
	230	439.2	57.5	17.2	17.7	3.0	22.87	69.4	21.4	3.6
Calorie controls	42	62.1	69.1	6.8	16.2	3.2	22.13	66.6	17.4	3.4
	110	119.0	66.7	8.6	18.3	4.0	24.14	73.0	20.0	4.4
	160	221.3	63.6	12.0	17.7	3.5	23.18	72.3	20.1	4.0
	230	289.3	61.3	13.6	18.4	3.6	23.83	71.0	21.3	4.2
Gliadin-stunted	42	46.5	66.3	11.6	15.1	3.4	20.83	75.0	17.1	3.9
	110	79.1	65.9	11.5	14.9	3.9	20.50	73.4	16.7	4.3
	160	187.1	62.4	13.8	17.2	3.5	22.30	72.4	20.0	4.1
	230	249.0	61.6	13.5	18.1	3.5	23.46	71.2	20.9	4.1

mentioned in the following discussion were found to be significant according to this treatment.

The rapidly growing and stock animals, fed similarly to the age of 42 days, showed no significant differences in composition at that age. At 110 days the former had a markedly greater fat content; at 230 days this difference though still present was somewhat reduced, owing chiefly to the increase in fat content of the stock rats.

The gliadin-stunted group contained at 42 days a proportion of fat comparable to that of the rapidly growing and stock rats. The calorie controls, as might be expected, contained little fat but proportionately more protein, resembling the two heavier groups in protein content. At 110 days both stunted groups differed strikingly from the rapidly growing animals in containing more water and less fat. No such replacement of fat by water was observed in gliadin-fed albino mice by Mendel and Judson ('15). Mice restricted in caloric intake, however, were found by these investigators to contain the same proportion of fat as younger normal mice of the same weight. Values relatively high for water and low for fat have been observed in animals stunted by other types of deficiencies (Mendel and Judson, '15; Light et al., '34; Johnson et al., '36). After realimentation the two stunted groups of this experiment approached the same composition but were still poorer in fat than the heavier groups.

Several investigators have commented upon various ratios and values for certain fractions in animal bodies. Moulton ('23), Armsby and Moulton ('25) and Johnson et al. ('36) have adduced evidence for the comparative constancy of the ratio of water to protein in animal bodies after chemical maturity (Moulton, '23) has been reached. Johnson and coworkers have calculated these values from the data furnished by other workers and have found that for mature rats reported averages vary from 2.89 to 3.95. In the present study the values for this ratio, which are derived from data shown in table 2, are indeed relatively constant, varying between 3.2 and 3.5 for "normally" and rapidly growing animals after chemical maturity (50 days of age) has been reached. The stunted animals during the period of inadequate nutrition showed high values for this ratio, a variation in the direction characteristic of younger animals. After realimentation this ratio changed in the direction of a normal value, but still remained slightly above that of the heavier groups.

The proportion of dry fat-free ash-free material in the bodies has been computed by Chanutin ('30) and by Light et al. ('34), the former reporting an average value of approxi-

mately 20%; the latter, 19.8%. Values derived from table 2 vary from 16.6% to 20.9% for mature animals of all dietary groups. The low figure refers to the gliadin-stunted group of 110 days; it varies in the same direction as the still lower figures for suckling young.

Armsby and Moulton ('25) calculated that the dry fat-free ash-free material contains approximately 16.05% of nitrogen. Chanutin ('30) and Light et al. ('34) reported this value as 15.9 and 15.3 respectively. Still lower figures found in the present investigation may be calculated from table 2. Averages for mature animals of all dietary groups vary from 14.3% to 14.8%; those for young animals on inadequate diets are slightly below 14.0%.

The variability of the fat content has led a number of investigators to express the composition of bodies on a fat-free basis. Murray ('19, '22) stated that the fat-free matter is of practically constant composition. Moulton ('23), by calculating values for the composition of bodies to the fat-free basis, was able to define regular and consistent changes with age. The group averages for the body composition of animals of this experiment, calculated to the fat-free basis, are included in table 2. Comparison with values for whole bodies makes clear the greater uniformity of composition expressed on this basis; most striking is the very close similarity of all four groups at 230 days.

Iodine number of body lipids. The iodine numbers of the body lipids are recorded in table 3. Obviously the most saturated fat was obtained from rats at birth. No significant differences appear in the average values for age groups of animals on different dietary regimens. These data do not illustrate the "hardening" of body fat with increasing weight (advancing age) observed by Anderson and Mendel ('28) in rats on a diet composed of two-thirds whole wheat and one-third dried whole milk. They suggest that rate of gain in body weight has little if any influence on the degree of unsaturation of the body lipids.

Composition of gains. Table 4 presents the percentage composition of the gains calculated from the group averages

TABLE 3

Iodine number of body lipids

EXPERIMENTAL GROUP	AGE (DAYS)	IODINE NUMBER (AVERAGE FOR GROUP)
	Birth	51.7
	14	64.1
	21	64.8
Rapidly growing	42	62.8
	110	67.5
	230	69.5
Stock	42	66.3
	230	69.3
Calorie controls	42	67.1
	110	69.6
	160	67.0
	230	68.5
Gliadin-stunted	42	70.1
	110	73.5
	160	65.7
	230	68.0

TABLE 4

Composition of gains

EXPERIMENTAL GROUP	PERIOD	GAIN IN NET WEIGHT	WATER	LIPIDS	PROTEIN	ASH
	Days	*gm.*	%	%	%	%
	0– 14	16.07	68.8	12.3	14.9	2.5
	14– 21	17.6	60.8	18.1	14.3	1.9
Rapidly growing	21– 42	122.0	63.2	14.0	17.5	2.2
	42–110	284.0	52.8	25.3	16.0	2.8
	110–230	95.0	36.5	41.6	13.9	3.2
Stock	21– 42	107.2	65.1	12.2	17.5	2.7
	42–110	203.2	59.2	14.9	21.3	3.7
	110–230	88.9	39.8	30.2	11.4	2.2
Calorie controls	21– 42	22.2	71.1	..[1]	20.7	5.3
	42–110	56.9	64.1	10.6	20.6	4.9
	110–160	102.3	60.0	16.0	17.0	2.9
	160–230	68.0	53.8	18.8	20.7	3.9
Gliadin-stunted	21– 42	6.6	56.2	1.8	23.6	11.4
	42–110	32.6	65.3	11.4	14.6	4.6
	110–160	108.0	59.8	15.5	18.9	3.5
	160–230	61.9	59.2	12.6	20.8	3.5

[1] The average lipid content in grams decreased during this interval.

for net weight and body composition. Mitchell and Carman ('26) have shown by computing the gains of individual rats that equal gains made at the same rate by litter-mates on the same diet may differ widely in composition. In the present experiment individual variation has been taken into account by the statistical treatment.

The trends found in the composition of bodies are magnified in the composition of gains. With a few exceptions, the proportion of fat increased and that of water declined with advancing age. No striking relationship is noted between age and proportion of protein or of ash in the gains.

Considered in terms of groups, these data on the gains show the tendency of the rapidly growing animals to accumulate greater proportions of fat and smaller proportions of water than the animals of any other groups. A tendency to higher values in protein content is found in the stunted animals. Among figures for ash content those for the stunted groups, especially during the period of inadequate feeding, are definitely high.

The results of this experiment relating to the fat content of the gains are in accord with those of Moulton, Trowbridge and Haigh ('22), which have been enhanced by Mitchell's ('29) mathematical treatment of the data. Steers were maintained at three planes of nutrition by different amounts of the same feed. It was found that the best nourished and most rapidly growing group added the greatest proportions of fat at all ages and the most slowly growing group contained and deposited the least fat. A similar statement holds for the two heavier groups of the present experiment. Contrary evidence was noted by Mitchell and Hamilton ('29) in hogs fed ad libitum a uniform diet. Individual variations in rate of gain were not paralleled by consistent differences in body composition.

Haecker ('20), who analyzed the bodies of steers over a wide range of weights, distinguished between two periods, the "growing stage," when protein exceeded fat in the gains, and the "fattening stage," when the reverse held. Similarly

Moulton and co-workers ('22) observed a "thinning" during early growth followed by a tendency to deposit fat after the period of most rapid gain in weight. They found that nitrogen storage followed that of water and was inversely proportional to fat deposition. With respect to the two adequately fed groups, the present experiment agrees with those of Haecker and of Moulton et al. Early rapid gains contained larger proportions of protein and smaller proportions of fat than later slower gains.

The stunted animals of the present experiment, however, experienced early slow growth and later rapid growth. The slow gains contained a preponderance of protein over fat, which was particularly striking during the first period studied. During realimentation, the period of most rapid growth of the stunted animals, the greatest percentage of fat appeared in their gains. Rate of gain in weight does not necessarily run parallel to preponderance of protein over fat in the gains.

One factor of importance is age; some of the earliest works on the composition of bodies brought out the influence of age on fatness. According to Reed, Yamaguchi, Anderson and Mendel ('30), fatty acid deposition is more closely correlated with body weight than with age. In the present experiment older stunted animals, however, made gains containing greater proportions of fat than did younger "normal" animals of the same body weight. The data might be interpreted to mean that in the early stages of "normal" growth the emphasis is placed upon increase of active body tissue, represented by protein, at the expense of fat deposition, whereas at mature ages the emphasis is on the accumulation of fat.

Another important influence is diet. This factor was not controlled for the rapidly and "normally" growing groups because it was desired to reproduce conditions studied previously. Whereas the caloric intakes of these two groups were similar, their diets differed in composition. The rapidly growing animals selected a mixture containing about 35% of protein and 35% of fat, while the "normal" group received about 18% of protein and 7% of fat. This difference in protein

levels may not have exerted a significant influence on the composition of the gains. Hamilton ('39) observed in controlled feeding experiments that differences in amount of whole egg protein ranging from 16% to 42% of the diet failed to cause marked differences in the composition of the gains. On the other hand, the difference in the amounts of lipids ingested undoubtedly affected the results of the present experiment. This is indicated by the work of Reed et al. ('30) and of Paquin ('35) which shows that dietary fat promotes fat deposition in rats to a greater extent than does carbohydrate. Since rate of gain is related to diet and since diet influences body composition, the relation between rate of gain and body composition is inevitably complicated.

The possibility of mild vitamin B_1 deficiency in the two stunted groups during the latter part of the period of realimentation cannot be overlooked. The work of McClure, Voris and Forbes ('34) has established the role of vitamin B_1 in promoting fat deposition. Even a degree of deficiency too mild to be detected by the growth curve was shown to interfere with the storing of fat. The tendency of the stunted animals to deposit more fat than protein during the period of their most rapid growth has probably not had full expression in this experiment. More striking results might have been obtained if an abundance of vitamin B_1 had been assured as the animals increased in size.

Early students suggested that the function of growth is retained only by exercising it, a conception soon disproved. In the present experiment those animals that achieved the most rapid early growth, in which water and protein were added in high proportions, accumulated in maturity disproportionate quantities of fat in the gains. In contrast, the stunted animals, given an opportunity for growth only at a mature age, made gains richer in protein and water than those made at the same age by the heavier animals. If previous performance has any effect on later growth, it appears that early suppression of growth may cause subsequent persistent

gains in lean tissue at the expense of some of the fat that is added in large amounts by normal animals of mature age.

SUMMARY AND CONCLUSIONS

Rates of gain including comparatively rapid early gain of rapidly growing and "normal" rats, the slow later increase of the same animals, the retarded early growth of two stunted groups and a more rapid increase in weight made by the stunted animals at a mature age, were produced by dietary adjustment.

Results relating to the rapidly growing and "normal" groups show that during early growth gains were rapid and contained a preponderance of protein over fat, while later gains were made considerably more slowly and were accompanied by the accumulation of large quantities of fat. The performance of the stunted animals, on the other hand, demonstrates conclusively that ratio of protein to fat in the gains does not, as has been suggested, run parallel to the rate of increase of body weight. During early life, when gains were slight, the stunted animals added much more protein than fat. After realimentation, when their most rapid increase in weight took place at a rate greater than that of the heavier groups of the same age, these animals added considerably more fat than previously. No single generality, therefore, relates rate of growth and composition of gains, but other factors such as age and nature of the diet appear to influence the composition of the gains.

The composition of the whole bodies of the four groups reflected the trends described for gains. The fat-free matter of the bodies showed smaller variations. At the end of the experiment, although the whole bodies differed considerably in size and composition, the fat-free material of the four groups was of practically identical composition.

The iodine number of the body fat rose sharply between the first and fourteenth days. Thereafter this value did not differ significantly for animals of different age and dietary history.

LITERATURE CITED

ANDERSON, W. E., AND L. B. MENDEL 1928 The relation of diet to the quality of fat produced in the animal body. J. Biol. Chem., vol. 76, p. 729.

ANDERSON, W. E., AND A. H. SMITH 1932 Further observations of rapid growth of the albino rat. Am. J. Physiol., vol. 100, p. 511.

ARMSBY, H. P., AND C. R. MOULTON 1925 The animal as a converter of matter and energy; a study of the role of live stock in food production. New York, The Chemical Catalog Co.

CHANUTIN, A. 1930 Studies on the creatinine and nitrogen content of the whole rat after the feeding of a variety of diets and after nephrectomy. J. Biol. Chem., vol. 89, p. 765.

CLARKE, M. F., AND A. H. SMITH 1938 Recovery following suppression of growth in the rat. J. Nutrition, vol. 15, p. 245.

HAECKER, T. L. 1920 Investigations in beef production. Univ. Minn. Agr. Exp. Sta. Bul. 193.

HAMILTON, T. S. 1939 The growth, activity, and composition of rats fed diets balanced and unbalanced with respect to protein. J. Nutrition, vol. 17, p. 565.

JACKSON, C. M., AND C. A. STEWART 1920 The effects of inanition in the young upon the ultimate size of the body and of the various organs in the albino rat. J. Exp. Zool., vol. 30, p. 97.

JOHNSON, S. R., A. G. HOGAN AND U. S. ASHWORTH 1936 The utilization of energy at different levels of protein intake. Univ. Mo. Agr. Exp. Sta. Res. Bul. 246.

LIGHT, A. E., P. K. SMITH, A. H. SMITH AND W. E. ANDERSON 1934 Inorganic salts in nutrition. XI. Changes in composition of the whole animal induced by a diet poor in salts. J. Biol. Chem., vol. 107, p. 689.

MAYNARD, L. A. 1930 Diet for stock rats. Science, vol. 71, p. 192.

MCCLURE, E. J., L. VORIS AND E. B. FORBES 1934 The utilization of energy producing nutriment and protein as affected by individual nutrient deficiencies. II. Effects of vitamin B deficiency. J. Nutrition, vol. 8, p. 295.

MENDEL, L. B., AND S. E. JUDSON 1915 Some interrelations between diet, growth, and the chemical composition of the body. Proc. Nat. Acad. Sci., vol. 2, p. 692.

MITCHELL, H. H. 1929 The minimum protein requirements of cattle. Report of Committee on Animal Nutrition. Bul. Natl. Res. Counc., no. 67.

MITCHELL, H. H., AND G. G. CARMAN 1926 The composition of the gains in weight and the utilization of food energy in growing rats. Am. J. Physiol., vol. 76, p. 398.

MITCHELL, H. H., AND T. S. HAMILTON 1929 Swine type studies. III. The energy and protein requirements of growing swine and the utilization of feed energy in growth. Ill. Agr. Exper. Sta. Bul. 323.

MOULTON, C. R. 1923 Age and chemical development in mammals. J. Biol. Chem., vol. 57, p. 79.

MOULTON, C. R., P. F. TROWBRIDGE AND L. D. HAIGH 1922 Studies in animal nutrition. III. Changes in chemical composition on different planes of nutrition. Mo. Agr. Exp. Sta. Res. Bul. 55.

MURRAY, J. A. 1919 Meat production. J. Agric. Sci., vol. 9, p. 174.

———— 1922 The chemical composition of animal bodies. J. Agric. Sci., vol. 12, p. 103.

OSBORNE, T. B., AND L. B. MENDEL 1919 The nutritive value of the wheat kernel and its milling products. J. Biol. Chem., vol. 37, p. 557.

PAQUIN, F., JR. 1935 The influence of arsenic and thyroglobulin on deposition and quality of fat, on growth, and on size of organs. Thesis. Yale University.

REED, L. L., F. YAMAGUCHI, W. E. ANDERSON AND L. B. MENDEL 1930 Factors influencing the distribution and character of adipose tissue in the rat. J. Biol. Chem., vol. 87, p. 147.

SNEDECOR, G. W. 1937 Statistical methods applied to experiments in agriculture and biology. Ames, Iowa, Collegiate Press, Inc.

THE INFLUENCE OF FAT ON CALCIUM AND PHOSPHORUS METABOLISM

JAMES H. JONES

Department of Physiological Chemistry, School of Medicine, University of Pennsylvania, Philadelphia

(Received for publication May 29, 1940)

Numerous reports have appeared during the past several years dealing with the influence of fat on the metabolism of calcium and phosphorus. Holt, Courtney and Fales ('20) found that the utilization of calcium by infants was augmented by fat in the diet. Hickmans ('24) has reported similar results. An increased absorption of calcium and phosphorus from the intestines of rats was observed by Boyd, Crum and Lyman ('32) when fat was added to the diet. Several investigators have studied the influence of fat on the development of rickets in rats. Zucker and Barnett ('23) have reported that cottonseed oil and its hydrogenated product [1] showed antirachitic properties although it was impossible to concentrate the antirachitic activity in the non-saponifiable fraction. From a series of investigations on butterfat, lard, egg oil and olive oil, and the fatty acids prepared from butter and lard, Kon and Booth ('33, '34 a, '34 b) have concluded that the antirachitic activity of the saponifiable fraction of a fat is due to the fatty acids and not to a specific compound. McDougall ('38) employing a diet deficient in calcium found that rats were protected against the low-calcium type of rickets by including 11% lard in the diet. Olive oil and cocoanut oil were less effective. Palmer and Mottram ('39) were unable to confirm the findings of McDougall. Gridgeman, Lees and

[1] Crisco.

Wilkinson ('39) observed that fat in the diet influenced the degree of new calcification produced by vitamin D.

In the following experiments the relation of fats to calcium and phosphorus metabolism has been further studied. Increased calcification in the long bones of rats on an otherwise rachitogenic diet has been used as a measure of the beneficial effect of the fats, and several of the possible modes of action suggested by the above-mentioned investigators have been tested. The investigations were facilitated by the use of synthetic rachitogenic diets in which it is possible to vary certain constituents of the diet without disturbing the quantitative relationships of the others. This is of particular importance in the study of rickets in the rat inasmuch as the ratio as well as the absolute amounts of calcium and phosphorus plays a significant role.

EXPERIMENTAL

Albino rats at approximately 25 days of age were placed on synthetic rachitogenic diets similar to those previously described (Jones, '39 a). The fat, or related substance, to be studied for its calcifying properties was added to the ration at the expense of the carbohydrate. In some experiments the substitution was made on a weight basis and in others on a calorie basis. With only a few exceptions, the basal diet without substitution was given to a group of rats as controls simultaneously with the feeding of the experimental rations to the test animals. The usual precautions were taken to distribute the animals with respect to sex and litter mates among the various groups. After about 21 days on the experiment the rats were bled and the pooled sera (usually from three animals) were analyzed for calcium by the method of Clark and Collip ('25) and for inorganic phosphorus on the calcium-free filtrate by the method of Gunther and Greenberg ('29). The right femurs were removed and the percentage of ash determined on the dry, lipoid-free bone. The wrists were removed for examination by the "line test" technic.

In table 1 are summarized the results of feeding various quantities of lard as a supplement to a rachitogenic diet. The first seven groups of animals were given diet no. 570 (Jones, '39 a) modified to the extent of reducing salt no. 5 to 5%.

TABLE 1

The effect of lard on bone ash and serum calcium and phosphorus of rats on rachitogenic diets

GROUP NO.	SALT NO.	LARD	NUMBER OF ANIMALS	FEMUR ASH		BLOOD SERUM		
						Number of determinations	Ca	P
				Yeast				
		%		*mg.*	%		*mg. per* 100 cc.	*mg. per* 100 cc.
1	5	0	23	20.0	25.0	8	12.0	2.9
2	5	5-W [1]	9	30.6	31.1	3	14.1	3.6
3	5	10-W	6	35.1	33.9	2	13.6	4.6
4	5	25-W	15	34.7	34.3	5	15.1	4.6
5	5	10-C [1]	6	32.8	34.1	2	14.0	4.7
6	5	25-C	3	38.0	37.5	1	14.0	7.0
7	5	50 [2]	3	20.8	23.1	1	12.0	2.0
8	10	0	13	21.9	26.0	4	11.3	3.8
9	10	10-W	3	34.7	33.6	1	13.3	5.3
10	10	25-W	3	39.2	35.2	1	13.7	6.1
				Liver extract + thiamin				
11	5	0	12	16.5	21.3	4	12.6	2.3
12	5	5-W	9	19.8	22.5	3	12.9	2.8
13	5	10-W	9	21.8	25.5	3	13.7	3.1
14	5	25-W	15	21.2	27.0	4	15.6	3.5
15	10	0	12	17.7	20.5	4	11.7	2.5
16	10	5-W	9	18.4	20.4	3	12.3	3.0
17	10	10-W	7	21.4	26.4	2	13.2	3.9
18	10	25-W	12	17.4	23.4	4	13.6	4.6

[1] See text for explanation.

[2] The non-saponifiable extract of the equivalent of 50% lard.

Diet no. 570 is composed of the following ingredients expressed in per cent: alcohol extracted fibrin 18; yeast 5; salt no. 5, 5.3; agar 2.0; carotene solution 0.1 and dextrin 69.6. In some of the experiments glucose [2] was used in place of the dextrin. This diet contains a little over 1% calcium and 0.09% phos-

[2] Cerelose.

phorus. The data obtained on the control animals (those receiving no lard) are summarized as averages and designated as group 1. The other groups received lard as indicated. The letter W following the amount of fat given denotes that an equal weight of carbohydrate was replaced by lard. The letter C indicates that an adjustment of the other constituents of the diet was made to allow for the increase in calories in the lard; that is, the lard still furnished a given portion of the diet by weight but all other constituents (except carbohydrate) were so increased at the expense of the carbohydrate as to keep unchanged the amounts of these constituents in unit calorie portions of the diet. The data show clearly that even as little as 5% of lard in the diet definitely increased calcification under these conditions. In general, in the animals receiving the lard there was an increase in both the absolute and relative amounts of ash in the femurs, and an increase in both the calcium and phosphorus of the serum. Examination of the distal end of the radii by the "line-test" technic also showed increased calcification. The bones were still not normal as revealed by this examination and could still be classed as rachitic, but the uncalcified areas were much narrower in the fat-fed animals than in the controls. Also the margins of these areas were less regular and the surrounding calcified portions appeared much denser. The results were the same whether the lard was given on a weight or calorie basis.

Group 7 was given, in addition to the basal diet, the unsaponifiable fraction of 50 gm. of lard per 100 gm. of diet, or an equivalent of 50% of the original fat. None of the criteria used in these studies indicated any increase in calcification as a result of this addition. This shows that the increased calcification produced by the lard was not due to the presence of vitamin D. Furthermore, as the lard contained only traces of phosphorus (approximately 0.002%) the results cannot be due to an increase of this element. The next three groups of animals (groups 8, 9 and 10) were given a basal diet containing 3% of salt no. 10 (Jones, '39 b). This salt mixture differs from salt no. 5 only in respect to its content

of calcium carbonate and when fed at a 3% level furnished about 0.38% calcium while the other ingredients of the salt mixture remained the same. Here again 10 and 25% of lard (the only two levels studied) definitely increased calcification.

In groups 11 to 18 inclusive, experiments very similar to those described above were repeated except that the vitamin B factors were supplied by liver extract [3] (usually at a 2% level) and 200 μg. of thiamin hydrochloride [4] per 100 gm. of diet. This change reduced the amount of phosphorus in the diet from about 0.09% to slightly more than 0.02%. As can be seen, the influence of fat on calcification was less marked when the phosphorus deficiency was made more severe. The serum calcium and phosphorus seem to have been affected by the lard to a greater extent than the bone ash especially when the fat was given at the 10 and 25% levels. Examination of the radii by the "line test" technic also indicated greater calcification than would have been predicted from the bone ash. This is in line with the recent report of Bunker, Harris and Mosher ('40) who found that the "line-test" and x-ray are more sensitive indicators of increased calcification than is the percentage of bone ash. Although no data are presented it was found that cottonseed oil and hydrogenated cottonseed oil [5] also definitely increased calcification. These two fats were fed at only the 25% level. Uncombined oleic acid [6] was about as effective as lard. On the other hand, the animals failed rapidly when 25% of stearic acid was included in the diet. Considerable amounts of this acid were excreted in the feces, partially in the form of the calcium soap. The loss of calcium was sufficient to produce a hypocalcemia with a concomitant rise in serum phosphorus. Several of these animals died within 3 weeks after being placed on the diet. Neither sodium oleate nor the calcium soaps of lard had any

[3] The author is grateful to Dr. C. E. Graham of The Wilson Laboratories, Chicago, Illinois, for supplying the alcohol-extracted fibrin and the liver extract used in these experiments.

[4] Kindly furnished by Merck and Co., Inc., Rahway, N. J.

[5] Crisco.

[6] U. S. P. Merck.

apparent effect on calcification under these conditions. In table 2 are summarized the results of an experiment in which seventeen rats were divided into five groups. One group was given the basal diet containing 5% of yeast and 5% of salt no. 5. The other groups were given supplements as indicated in the table. All supplements were fed at a 10% level with adjustments of other constituents on a calorie basis as described above. In the diet containing the calcium soaps of lard the calcium carbonate of the salt mixture was decreased to an extent equivalent to the amount of the calcium added as the soaps, thus preventing any change with respect to this element. As judged from bone ash the lard and oleic acid

TABLE 2

A comparison of the effects of lard, oleic acid, sodium oleate and calcium soaps of lard on bone ash and serum calcium and phosphorus

SUPPLEMENTS TO BASAL DIET	NUMBER OF ANIMALS	BONE ASH		BLOOD SERUM	
				Ca	P
		mg.	%	*mg. per* 100 cc.	*mg. per* 100 cc.
None	3	18.3	24.6	12.2	2.9
Lard	3	33.8	36.2	15.0	5.8
Oleic acid	3	26.4	31.2	15.0	3.1
Na oleate	4	19.6	25.3	11.4	3.3
Ca soaps	4	17.9	24.3	14.1	3.3

definitely increased calcification, whereas sodium oleate and the calcium soaps of lard were without effect. There is some discrepancy in the calcium and phosphorus values of the serum. It appears that the oleic acid failed to increase the level of phosphorus whereas the calcium soaps increased serum calcium. The compositions of the sera of these two groups are, therefore, almost identical, although there was greater calcification in the former than in the latter case. However, no significance can be attached to single determinations as there is considerable individual variation in the concentration of these elements and the values are dependent on several factors. The averages of two other determinations on sera from animals receiving oleic acid were: Ca, 13.0 and P, 4.2 mg.

per 100 cc. Increased calcification in the animals receiving the lard and oleic acids as compared to the other groups in the experiment was also indicated by examination of the wrist bones according to the "line-test" technic. Several additional experiments have confirmed the above observation that under the prevailing conditions, oleic acid definitely shows antirachitic properties whereas sodium oleate and the calcium salts of lard do not.

DISCUSSION

In the light of the data presented above it is now possible to reconsider some of the theories advanced as possible explanations for the manner in which fat increases the utilization of calcium and phosphorus. Zucker and Barnett ('23) believe that a fat acts as an antirachitic by uniting with the calcium to form soaps of the fatty acids and thus liberating a larger portion of the phosphorus. If that were the case some antirachitic effect should have been obtained by simply decreasing the amount of calcium in the diet. Direct comparisons were made between salt no. 5 at a 5% level (1.1% calcium) fed with 10% lard and salt no. 10 at a 3% level (0.38% calcium) fed with no lard. Assuming the average molecular weight of the fatty acids of lard to be equal to that of oleic acid and that all of the acids united with calcium, 10% of lard would combine with only 0.7% of calcium. There is thus about 0.4% of calcium still available to combine with the phosphorus. This is equal to the amount in the diet containing salt no. 10 and no lard. Without exception the greater calcification took place on the diet containing the lard. On the other hand, when the calcium soaps of lard were fed and the calcium of the basal diet was adjusted to allow for the added calcium, rickets was produced. These results seem to disprove the theory of Zucker and Barnett. According to McDougall's ('38) idea, fats act by forming the calcium soaps and by so doing increase the absorption of this element. The experiment with the calcium soaps just referred to apparently contradicts this theory.

According to the opinion expressed by Gridgeman, Lees and Wilkinson ('39) fats act indirectly by lowering the consumption of salts. The experiments of these authors are open to criticism as a constant intake of calcium and phosphorus was not maintained when the fat was added to the diet. It is in experiments of this kind that a basal diet composed of individually purified materials can be employed to considerable advantage. As described above, experiments were performed in which the lard was substituted for the relatively pure carbohydrate on both a weight and calorie basis. In both types of experiments the ratio of calcium to phosphorus remained unchanged, and in the latter the amounts consumed varied only as the intake of calories varied. Although daily food consumption was not measured there was no obvious increase or decrease in the amount of food eaten when fat was added to the diet. In a few cases the rats getting the fat gained slightly more weight than their controls, but usually there was no significant difference in this respect. It is, consequently, improbable that in these experiments the antirachitic action of the lard was the result of an altered intake of either calcium or phosphorus.

Boyd, Crum and Lyman ('32) explain the action of fat on calcification on the basis that it increases the acidity of the intestinal tract with subsequent greater absorption of calcium and phosphorus. The fact that lard and oleic acid increase calcification while sodium oleate and the calcium salts of lard do not is suggestive evidence in favor of this view. The results of experiments in progress at the present time, in which the effect of various calcifying agents on the hydrogen ion concentration of the intestinal tract is being studied, will be reported later.

Regardless of the mode of action, the above data show clearly that, aside from their content of vitamin D, fats exert a definite calcifying action in the rat. The response of the rat to the various factors influencing calcium and phosphorus utilization is somewhat different from that of most mammals. Whether this difference holds for fats remains to be deter-

mined. If there should be a variation in this respect the antirachitic value of high fat-low vitamin D foods as determined on the rat may not be entirely applicable to other animals. Lard, to the extent of only 5% of the diet, showed definite antirachitic properties especially when given in conjunction with the basal diet containing the higher amount of phosphorus. Whole corn contains nearly this much fat. It is possible that the occasional difficulties encountered in the production of rickets on the Steenbock-Black ('25) diet may be due in part to an exceptionally large amount of fat in the particular sample of corn being used.

SUMMARY

Lard when added to synthetic rachitogenic diets in amounts varying from 5 to 25% had definite antirachitic properties which were not associated with the non-saponifiable fraction. The lard increased calcification on diets containing excessive (1.1%) or moderate (0.38%) amounts of calcium. The lard was effective when added on either a weight or calorie basis. The effect of the lard was less pronounced on diets very low in phosphorus (0.02%) than on diets higher in phosphorus (0.09%). Oleic acid also increased calcification but sodium oleate and the calcium soaps of lard showed no antirachitic action. These data are discussed in relation to the various theories advanced to explain the action of fats in favorably influencing calcium and phosphorus utilization. With the exception of the effect on the acidity of the intestinal tract these theories are apparently disproved by the data here reported.

LITERATURE CITED

BOYD, O. F., C. L. CRUM AND J. F. LYMAN 1932 The absorption of calcium soaps and the relation of dietary fat to calcium utilization in the white rat. J. Biol. Chem., vol. 95, p. 29.

BUNKER, J. W. M., R. S. HARRIS AND L. M. MOSHER 1940 Relative efficiency of active wave lengths of ultraviolet in activation of 7-dehydrocholesterol. J. Am. Chem. Soc., vol. 62, p. 508.

CLARK, E. P., AND J. B. COLLIP 1925 A study of the Tisdall method for the determination of blood serum calcium with a suggested modification. J. Biol. Chem., vol. 63, p. 461.

GRIDGEMAN, N. T., H. LEES AND H. WILKINSON 1939 The biological vitamin D assay of low-potency materials with special reference to the role of the mineral content of the diet. Biochem. J., vol. 33, p. 645.

GUNTHER, L., AND D. M. GREENBERG 1929 A note on the determination of the inorganic phosphate of the serum on the filtrate from calcium analysis. J. Biol. Chem., vol. 82, p. 551.

HICKMANS, E. M. 1924 The calcium metabolism of atrophic infants and its relationship to their fat metabolism. Biochem. J., vol. 18, p. 925.

HOLT, L. E., A. M. COURTNEY AND H. L. FALES 1920 Calcium metabolism of infants and young children, and the relation of calcium to fat excretion in the stools. Am. J. Dis. Children, vol. 19, p. 97.

JONES, J. H. 1939 a A study of rachitogenic diets composed of purified food materials. J. Nutrition, vol. 17, p. 601.

———— 1939 b A comparison of cereal and non-cereal diets in the production of rickets. J. Nutrition, vol. 18, p. 507.

KON, S. K., AND R. G. BOOTH 1933 The vitamin D activity of butter. I. A chemical differentiation of the antirachitic factor of autumn and winter butter from irradiated ergosterol and the vitamin D of cod-liver oil. Biochem. J., vol. 27, p. 1302.

———— 1934 a The vitamin D activity of butter. II. The complex nature of the antirachitic action of butter. Biochem. J., vol. 28, p. 111.

———— 1934 b The vitamin D activity of butter. III. An attempt to elucidate the nature of the labile factor in butter antirachitic for the rat. The antirachitic potency of lard, olive oil, egg oil and the fatty acids of butter and lard. Biochem. J., vol. 28, p. 121.

MCDOUGALL, E. J. 1938 The counteraction by fat of the anticalcifying action of cereals. Biochem. J., vol. 32, p. 194.

PALMER, N., AND J. C. MOTTRAM 1939 The influence of phytin and of fats on the production of rickets by a cereal diet. Biochem. J., vol. 33, p. 512.

STEENBOCK, H., AND A. BLACK 1925 Fat-soluble vitamins. XXIII. The induction of growth-promoting and calcifying properties in fats and their unsaponifiable constituents by exposure to light. J. Biol. Chem., vol. 64, p. 263.

ZUCKER, T. F., AND M. BARNETT 1923 Observations on the distribution of antirachitic substances. Proc. Soc. Biol. Med., vol. 20, p. 375.

THE INFLUENCE OF DIETARY RIBOFLAVIN ON THE CONTENT OF THIS VITAMIN IN CHICKEN TISSUE

A. Z. HODSON

Department of Poultry Husbandry, Cornell University, Ithaca, New York

(Received for publication June 29, 1940)

Several reports have been made on the riboflavin or vitamin G content of meats. Many of the earlier attempts to quantitatively measure this vitamin in meat and meat products were measurements of a part of the vitamin B complex rather than a specific measurement of riboflavin. The isolation and synthesis of riboflavin made possible new and better methods for the determination of riboflavin. In 1938 Darby and Day reported the riboflavin content of meat as determined by the rat growth method. In 1939 Mickelsen, Waisman and Elvehjem made a more extensive study of the same problem by use of a microbiological method of measurement. The results of these two studies are in good agreement and are comprehensive enough to give a fairly complete picture of the riboflavin content of the meats which are used for human food. However, these reports have not included any attempt to study the factors affecting the riboflavin content of meat. The development of the microbiological method of Snell and Strong ('39) and the fluorometric method of Hodson and Norris ('39) for determining riboflavin have made possible studies of this character, since these methods are rapid, accurate, and require only a small amount of material as a sample. The microbiological method has been used by Fraser, Topping and Isbell ('40) to study variations in the riboflavin content of tissues of rats and dogs fed diets deficient and adequate in this vitamin. Since neither of these species is of economic importance as a source of meat, a further investigation has been undertaken.

The chicken has been used for this investigation since riboflavin is very important for this species as pointed out by Norris and others ('36) and by Schumacher and Heuser ('39), and because the chicken is small in size it can be kept under controlled dietary and environmental conditions at a small cost. While the results obtained in such an experiment are primarily applicable only to the one species, they also serve as pilot observations suggesting work with other animals.

EXPERIMENTAL

Groups of Single Comb White Leghorn chicks were placed both on a diet deficient in riboflavin and on one containing an excess of this vitamin, and the riboflavin content of the tissues and organs was determined. The percentage composition of the basal diet was as follows: table corn meal 55.5, peanut oil meal 15.0, casein 11.0, cane molasses 5.0, salt mixture 5.0, soybean oil 3.0, fortified cod liver oil 0.5, and wheat bran 5.0. The diet for the group of chicks receiving an excess of riboflavin was composed of the basal ration 95% and yeast 5%. To each gram of feed 4.34 μg. of synthetic riboflavin were added. Analyses indicated that the diet contained 6.9 μg. of riboflavin per gram. The diet deficient in riboflavin was composed of 99% basal diet and 1% of yeast. Analyses indicated that this ration contained 1.0 μg. of riboflavin per gram.

The chicks were maintained on the diets for 6 weeks and were then killed and the tissues removed. The samples were weighed, dried in hot air at 50 to 60°C. for 24 hours, reweighed and ground. The riboflavin content of the tissues and feed was determined by the fluorometric method of Hodson and Norris ('39). Samples of liver, heart, gizzard, leg, and breast from the two sources were compared. In addition riboflavin determinations were made on two samples of spleen, two samples of eye, and one sample each of lung, comb and brain from the chickens receiving the excess of riboflavin. The entire liver, gizzard, heart, eye, spleen, brain, comb or lung was used for a sample. In a few cases organs from two or more birds were combined to form a sample. The hearts from chickens on the

low riboflavin diet were very small and were, therefore, combined to form a single sample. The breast samples were from a single bird. The leg samples consisted of all the muscular tissue surrounding the femur and tibia on one leg of a bird and constituted the cuts usually designated as the thigh and drumstick. The breast samples represented light meat and the leg samples dark meat.

The results which are presented in table 1 indicate that the amount of riboflavin in the diet of chickens has a marked influence on the riboflavin content of their tissues and organs. The tissues and organs of the birds fed a diet containing an excess of riboflavin contained from two to four times as much riboflavin as those from birds fed a ration deficient in this factor. The riboflavin content of a single tissue from chickens on the same diet also shows some variation (table 1). This individual variation is the most marked in the case of the leg tissue. However, the riboflavin content of the leg samples from the chickens on the excess riboflavin diet is as high or higher than that reported for other muscular meats by Mickelsen, Waisman and Elvehjem ('39). In agreement with their data the results presented in table 1 indicate that the white breast meat has a very low riboflavin content. Riboflavin determinations on tissue from five birds fed a commercial diet indicated that the leg tissue and breast tissue contained respectively 9.3 μg. and 3.6 μg. per gram of dry tissue. These results are also in excellent agreement with those reported by Mickelsen, Waisman and Elvehjem for chicken meat from birds on a normal ration.

The results of determinations on two samples of spleen, two samples of eye, and one sample each of lung, comb, and brain, indicated that these tissues contained 23.2, 16.4, 26.5, 6.4 and 28.0 μg. respectively of riboflavin per gram of dry tissue.

The fact that riboflavin is necessary in the diet of chickens not only to provide for the growth and health of the fowl, but also to assure that the muscle becomes a good source of riboflavin is important, since there is an increasing amount of

TABLE 1

The influence of diet on the riboflavin content of the tissue of chickens

TISSUE [1]	LOW RIBOFLAVIN DIET Riboflavin content of		HIGH RIBOFLAVIN DIET Riboflavin content of	
	Fresh tissue	Dry tissue	Fresh tissue	Dry tissue
	μg./gm.	*μg./gm.*	*μg./gm.*	*μg./gm.*
Liver	9.9	43	42.6	129
Liver	9.8	35	31.8	112
Liver	12.2	46	32.3	119
Liver	12.0	41	34.8	118
Liver	12.3	44	32.9	113
Liver	10.0	35	32.7	112
Liver	11.3	39	32.9	112
Liver	11.9	44	28.2	95
Liver	10.5	39	29.2	94
Liver	12.2	46	31.4	110
Liver average	11.2	41	32.9	111
Heart	4.3 [2]	19.4	18.4	71.2
Heart			20.4	80.4
Heart			16.7	63.0
Heart			18.4	72.3
Heart			19.5	72.6
Heart average	4.3	19.4	18.4	71.2
Gizzard	0.9	3.5	4.3	15.2
Gizzard	1.4	5.4	3.9	14.5
Gizzard	1.5	5.7	4.4	14.9
Gizzard	1.1	4.2	4.0	13.5
Gizzard	1.4	5.6	4.0	14.3
Gizzard average	1.3	4.9	4.1	14.5
Leg	1.0	4.3	4.6	18.8
Leg	1.1	4.7	4.1	15.6
Leg	1.0	4.8	7.4	29.3
Leg	0.7	3.5	5.6	21.3
Leg	0.8	3.2	6.0	24.0
Leg	1.0	4.4	3.4	14.5
Leg	1.0	4.3	2.4	10.1
Leg	1.0	4.7	3.1	13.0
Leg	1.1	3.8	3.2	13.3
Leg	1.0	4.7	2.5	10.6
Leg average	1.0	4.2	4.2	17.0
Breast	0.4	1.9	0.8	2.8
Breast	0.5	2.3	0.7	2.9
Breast	0.5	2.4	0.8	2.8
Breast	0.4	1.8	0.9	3.4
Breast	0.4	1.7	0.9	3.8
Breast average	0.4	2.0	0.8	3.1

[1] Each sample of a tissue is from one or more different chickens.

[2] Composite sample of hearts of five chickens.

evidence that riboflavin is necessary in human nutrition and that cases of ariboflavinosis often occur in the United States. This has been shown by Sebrell and Butler ('38, '39), Oden et al. ('39), Jolliffe and others ('39) and Kruse and associates ('40).

The effect of dietary riboflavin on the content of this vitamin in meat may not be limited to fowl but may also be important in certain other species such as swine, which are grown primarily for their meat. This question is one which appears worthy of further research. It has been reported by McElroy and Goss ('39) that dietary riboflavin is probably less important in the case of ruminants, as apparently bacteria in the rumen synthesize riboflavin.

SUMMARY

1. The amount of riboflavin in the diet has a marked effect on the riboflavin content of the tissues of chickens.

2. Of the tissues examined liver proved to be richest in riboflavin content; heart was next; gizzard and leg muscle were next and were about equal in value; breast muscle was poorest of all.

LITERATURE CITED

DARBY, W. J., AND P. L. DAY 1938 Riboflavin content of meats. J. Nutrition, vol. 16, pp. 209–218.

FRASER, H. F., N. H. TOPPING AND H. ISBELL 1940 The bacterial assay of riboflavin in the urine and tissues of dogs and rats. U. S. Public Health Reports, vol. 55, pp. 280–289.

HODSON, A. Z., AND L. C. NORRIS 1939 A fluorometric method for determining the riboflavin content of foodstuffs. J. Biol. Chem., vol. 131, pp. 621–630.

JOLLIFFE, NORMAN, HARRY D. FEIN AND LOUIS A. ROSENBLUM 1939 Riboflavin deficiency in man. New England J. Med. vol. 221, pp. 921–926.

KRUSE, H. D., V. P. SYDENSTRICKER, W. H. SEBRELL AND H. M. CLECKLEY 1940 Ocular manifestations of ariboflavinosis. U. S. Public Health Reports, vol. 55, pp. 157–169.

MCELROY, L. W., AND HAROLD GOSS 1939 Report on four members of the vitamin B complex synthesized in the rumen of the sheep. J. Biol. Chem., vol. 130, pp. 437–438.

MICKELSEN, OLAF, HARRY A. WAISMAN AND C. A. ELVEHJEM 1939 The distribution of riboflavin in meat and meat products. J. Nutrition, vol. 18, pp. 517–526.

NORRIS, L. C., H. S. WILGUS, JR., A. T. RINGROSE, VICTOR HEIMAN AND G. F. HEUSER 1936 The vitamin G requirement of poultry. Cornell University Agricultural Experiment Station Bulletin 660.

ODEN, J. W., L. H. ODEN AND W. H. SEBRELL 1939 Report of three cases of ariboflavinosis. U. S. Public Health Reports, vol. 54, pp. 790–792.

SCHUMACHER, A. E., AND G. F. HEUSER 1939 The importance of riboflavin in reproduction in poultry. Poultry Science, vol. 18, pp. 369–374.

SEBRELL, W. H., AND R. E. BUTLER 1938 Riboflavin deficiency in man, a preliminary note. U. S. Public Health Reports, vol. 53, pp. 2282–2284.

———— 1939 Riboflavin deficiency in man. U. S. Public Health Reports, vol. 54, pp. 2121–2131.

SNELL, E. E., AND F. M. STRONG 1939 A microbiological assay for riboflavin. Ind. and Eng. Chem. Anal. Ed., vol. 11, pp. 346–350.

THE COMPARATIVE ASSIMILATION OF FLUORINE BY GROWING RATS DURING CONTINUOUS AND INTERMITTENT DOSAGE [1,2]

MARGARET LAWRENZ, H. H. MITCHELL AND W. A. RUTH
Division of Animal Nutrition, and Department of Horticulture, University of Illinois, Urbana

(Received for publication July 2, 1940)

In studying the nutritional effects of nutrients, or the physiological effects of deleterious dietary constituents, by feeding experiments on animals, the customary procedure is to feed the nutrient or dietary constituent as a constant proportion of a suitably compounded ration. This is a systematic procedure that commends itself to most nutritionists and physiologists. However, in practical nutrition, at least in human nutrition, constancy in diet is the exception rather than the rule. It becomes a problem, therefore, whether the reaction of the body to the irregular intake of a dietary constituent is the same as the reaction to a regular intake. In particular, can the reaction to an irregular intake be predicted from the known results of a regular intake?

One possible cause of a quantitative difference in the physiological results of ingesting a given amount of a dietary constituent over a period of time, first, as a constant component of the diet, and, second, as an irregular component, is the interdependence in metabolism of the constituent in question

[1] This experiment was made possible by the donation of funds to the University of Illinois by the Aluminum Company of America and the Pennsylvania Salt Manufacturing Company.

[2] This investigation was conducted under the supervision of a Committee on the Physiological Effects of Spray Chemicals, appointed by the director of the Agricultural Experiment Station and consisting of the following members: H. H. Mitchell, W. A. Ruth, W. P. Flint and Julia P. Outhouse.

and one or more of the other components of the diet. Thus, the maximum effect of carbohydrate on protein metabolism is dependent upon the concurrent feeding of the two nutrients (Larson and Chaikoff, '37), while a demonstrable effect is dependent upon feeding them separately at intervals no greater than 4 hours in the case of canine subjects. Similarly, the favorable effects on experimental animals of a tryptophane supplement to a tryptophane-deficient diet are observed only when the supplement is fed concurrently with the basal diet, or at a time not too far removed from meal time (Berg and Rose, '29; Elman, '39).

The time relations in the interdependent action of two dietary components evidently depend upon the ability of the body to retain in its tissues in an unmetabolized form the component consumed first. This retaining ability will vary with the nature of the dietary component, and for the same component, it seems to vary also with the species of animal. Thus, the chick apparently stores vitamin A much less efficiently (and hence less readily) than the rat (Vermès, Lissot, Meunier and Raoul, '39). In the case of dietary constituents the physiological disposal of which is dependent upon other dietary components, it may be expected that the results of intermittent or irregular feeding may differ most from the results of continuous feeding when the storage ability of the organism is least; also, that, when a difference develops, the smaller degree of assimilation in the body will be associated with intermittent feeding. Furthermore, and for the same reasons, one might expect that, if two dietary constituents, the disposals of which in metabolism are to some extent interdependent, are consumed together, the metabolism of each will be modified to a different extent if the ratio of one to the other is changed.

When dietary components are stored in the body as a result of continuous feeding, the stores are subject to accretion only as the regime continues. But when storage occurs as a result of intermittent feeding, the stores would be subject both to accretion immediately following dosage, and to depletion by metabolism during the intervals between dosages. If the rate

of metabolism of the stored dietary component is greater than the rate of its metabolism when circulating in the body fluids and tissues, intermittent feeding again would be associated with less efficient utilization than would continuous feeding. A case in point is vitamin A, the reserves of which in the body are depleted at a rate directly proportional to their size (Baumann, Riising and Steenbock, '34), and, for the larger storages, at rates exceeding the day-to-day requirement. For this reason, large single doses of vitamin A or carotene are less effective nutritionally than small daily doses (Goss and Guilbert, '39). Apparently for a similar reason, massive doses of liver extract given at intervals of several months to patients with pernicious anemia may be less effective in prolonging the period of remission than small doses given at much shorter intervals of time (Strauss and Pohle, '40). The fact that the efficiency of calcium utilization by the growing rat is less the greater the storage of calcium in the body (Rottensten, '38) would seem also to have a bearing on this problem.

The consumption of sprayed fruits and vegetables in human nutrition is irregular, not only with reference to season, but undoubtedly also during periods when such foods are readily available. The ingestion of spray chemicals will also be irregular. Hence, their physiological effects may not be predictable, for the reasons just considered, from the results of feeding experiments involving the continuous administration of the chemical studied.

The purpose of the experiment to be described below is to study the comparative retention of fluorine in the spray chemical cryolite (synthetic) when offered to growing rats, first, as a constant component of the diet, and, second, as an irregular component of the diet; specifically, cryolite was added to the basal diet every third day throughout the feeding period. Comparative animals received the same total amount of fluorine. The concentration of fluorine in the total consumed food was purposely kept low, averaging only 7.34 p.p.m., in order that the results obtained would be more significant to the spray-residue problem.

EXPERIMENTAL METHODS

Twelve pairs of littermate rats were fed a basal ration consisting of the following ingredients, mixed in the indicated proportions: dried unextracted egg 30, dried extracted egg 7, ground yellow corn 55, dried yeast 3, fortified cod liver oil 2, fluorine-free salt mixture[3] 2, and calcium carbonate 1% respectively. This ration contained 1.4 p.p.m. of fluorine and was fed in equal amounts to pair mates. In addition to the basal diet, to which the rats had access continuously, or until the day's portion was consumed, one rat in each pair received daily 1 cc. of an aqueous solution of synthetic cryolite, containing 18 p.p.m. of fluorine, for each 3 gm. of food weighed out. The other rat in the pair received on every third day of feeding as much of the cryolite solution as its pair mate was given on that and the 2 preceding days. Thus, the total intakes of fluorine by pair mates were equalized every 3 days. The portions of the cryolite solution allotted to the rats were measured from a burette calibrated to 0.1 cc. and were mixed with the day's food. The solution was renewed at frequent intervals to insure against possible deterioration, and pair mates received the same volume of solution from each make.

After the sixth week of experimental feeding, the teeth of the rats were examined once a week for the appearance of striations, using a jeweler's lens with a magnification of 4 $\times$.

The experiment was terminated and the rats sacrificed generally at the end of 20 weeks. Three pairs were killed at the termination of 15 weeks, while with one pair the experiment had to be concluded after 88 days on account of the death of one of the rats, receiving continuous fluorine dosage, from a respiratory infection.

The rats were killed with ether and the empty weights and body lengths taken. The carcasses were then autoclaved and the flesh, bones and teeth separated and analyzed for fluorine by methods previously described (Lawrenz, Mitchell and Ruth, '39).

[3] Wesson.

EXPERIMENTAL RESULTS

Striations of the teeth were slow in appearing because of the low concentration of fluorine in the diets. Some rats failed to show striations even after 16 weeks of feeding, and among those developing definite striations no distinction could be made between those receiving a continuous dosage of fluorine and those receiving an intermittent dosage.

TABLE 1

Average data for all twelve pairs of experimental animals

	FLUORINE DOSAGE	
	Daily	Intermittent
Initial body weight, gm.	45	45
Total gain in weight, gm.	247	251
Final body length, mm.	232	233
Total food consumed, gm.	1264	1264
Number of food refusals	17	17
Total intake of fluorine, mg.	9.280	9.280
Feeding period, days	133	133
Final empty body weight, gm.	292	296
Weight of dry fat-free bone, gm.	13.6121	13.4315
Weight of dry teeth, gm.	0.4744	0.4694
Weight of soft tissues, gm.	267.5	269.7
Fluorine in bones, p.p.m.	224	212
Fluorine in bones, mg.	3.039	2.832
Fluorine in teeth, p.p.m.	116	112
Fluorine in teeth, mg.	0.055	0.053
Fluorine in soft tissues, p.p.m.	0.29	0.26
Fluorine in soft tissues, mg.	0.081	0.073
Total fluorine in carcass, mg.	3.175	2.957
Initial fluorine content, mg.	0.235	0.235
Total fluorine retained, mg.	2.940	2.722
Total fluorine retained, %	32.11	29.64

To conserve space, the growth data and the chemical data of the experiment are presented in average form in table 1. Each figure in the table is an average of twelve rats. Exclusive of the cryolite solutions, seventy-three samples were analyzed for fluorine.

The rats consumed an average of 1264 gm. of food and 9.280 mg. of fluorine, or 7.34 p.p.m. Nineteen per cent of the fluorine intake was, on the average, contained in the basal diet.

In an average period of 133 days the rats gained about 250 gm. in body weight, or 1.88 gm. per day.

Statistical analysis of the paired data of the experiment by the method of Student ('25) reveals no significant differences between continuous and intermittent feeding of fluorine with reference to gain in body weight, attained body length, weight of dry fat-free bone, dry weight of teeth or weight of soft tissue.

For the fluorine data, the statistics summarized in table 2, were obtained.

TABLE 2

The statistical significance of pair differences

ITEM	MEAN DIFFERENCE [1]	STANDARD DEVIATION OF DIFFERENCES	PROBABILITY
Fluorine in bones, p.p.m.	11.83	11.49	0.0029
Fluorine in bones, mg.	0.208	0.313	0.025
Fluorine in teeth, p.p.m.	4.33	8.49	0.060
Fluorine in teeth, mg.	0.00258	0.00545	0.073
Fluorine in soft tissues, p.p.m.	0.0283	0.1074	0.20
Fluorine in soft tissues, mg.	0.0080	0.0320	0.21
Total fluorine retained, mg.	0.217	0.237	0.0057

[1] A positive mean difference signifies that the mean for the continuous dosage rats exceeds the mean for the intermittent dosage rats.

All of the average differences between paired rats favored the rats receiving the continuous dosage of fluorine, i.e., for all samples and for the entire carcass, the average concentration of fluorine and the average total content of fluorine was greater for the continuous dosage rats. However, the average differences relating to the teeth and the soft tissues, both of which contained less than 5% of the fluorine in the carcass, were not significant statistically, as indicated by the probability values given in the last column of the table. These values express the probability on a scale of 1 that average differences as great or greater than those obtained, respectively, would have resulted from the operation of fortuitous factors only. If the probability is 0.03 or less, it may reasonably be neglected and the conclusion drawn that the corresponding average difference is the direct result, in part at

least, of the deliberately imposed difference in experimental treatment, namely, the method of administering fluorine.

From the statistical analysis, therefore, it may be concluded that the continuous feeding of fluorine has resulted in a greater retention of the element in the carcasses of the rats, and specifically in the bones, than the intermittent feeding of the same amount of fluorine. Under the conditions of this experiment, 7.4% less fluorine was retained when administered intermittently (every third day) than when administered daily.

The application of this finding to the spray residue problem would seem to be that the physiological effects of cryolite residues on sprayed fruits or vegetables should be somewhat less under conditions of practical nutrition than would be expected from experiments involving constant daily dosage.

CONCLUSIONS

1. The continuous administration of fluorine as synthetic cryolite to growing animals results in a greater retention of fluorine in the bones, and possibly in the teeth, than the intermittent administration of the same quantity of the element.

2. Hence, in practical human nutrition, which involves variable and intermittent consumption of foods, the physiological effects of a given dosage of fluorine in spray residues on fruits or vegetables may be expected to be somewhat less than that predicted from experiments involving the continuous administration of fluorine.

LITERATURE CITED

BAUMANN, C. A., B. M. RIISING AND H. STEENBOCK 1934 Fat-soluble vitamins. XLII. The absorption and storage of vitamin A in the rat. J. Biol. Chem., vol. 107, pp. 705–715.

BERG, C. P., AND W. C. ROSE 1929 Tryptophane and growth. 1. Growth upon a tryptophane-deficient diet supplemented at varying intervals by the separate feeding of tryptophane. J. Biol. Chem., vol. 82, pp. 479–484.

ELMAN, R., WITH THE TECHNICAL ASSISTANCE OF R. CHARNAS 1939 Time factor in retention of nitrogen after intravenous injection of a mixture of amino acids. Proc. Soc. Exptl. Biol. and Med., vol. 40, pp. 484–487.

GOSS, H., AND H. R. GUILBERT 1939 The minimum vitamin A and carotene requirement of the rat. J. Nutrition, vol. 18, pp. 169–179.

LARSON, P. S., AND I. L. CHAIKOFF 1937 The influence of carbohydrate on nitrogen metabolism in the normal nutritional state. J. Nutrition, vol. 13, pp. 287–304.

LAWRENZ, M., H. H. MITCHELL AND W. A. RUTH 1939 The comparative toxicity of fluorine in calcium fluoride and in cryolite. J. Nutrition, vol. 18, pp. 115–125.

ROTTENSTEN, K. V. 1938 The effect of body stores on the efficiency of calcium utilization. Biochem. J., vol. 32, pp. 1285–1292.

STRAUSS, M. B., AND F. J. POHLE 1940 The duration of remission in pernicious anemia with liver therapy. The efficacy of massive doses administered at one time. J. Amer. Med. Assn., vol. 114, pp. 1318–1320.

STUDENT 1925 New tables for testing the significance of observations. Metron, vol. 5, pp. 105–120.

VERMÈS, M., G. LISSOT, P. MEUNIER AND Y. RAOUL 1939 Sur la rapidité de la carence en vitamine A chez le poussin. Comp. rend. acad. agr., France. vol. 25, pp. 1090–1092.

AVAILABILITY TO WHITE RATS OF PHOSPHORUS IN SOYBEAN AND RED CLOVER HAYS

DOROTHY E. WILLIAMS, FLORENCE L. MacLEOD AND ELISE MORRELL
Department of Home Economics, Agricultural Experiment Station, University of Tennessee, Knoxville, in cooperation with The Tennessee Valley Authority

(Received for publication July 12, 1940)

THE PROBLEM

This paper represents a continuation of the studies already published ('40) comparing the availability to white rats of the phosphorus of a low phosphorus hay and of a high phosphorus hay. The types of hay used in this work were soybean and red clover.

EXPERIMENTAL PROCEDURE

Rapidly-growing white rats, 30 to 31 days old, and weighing from 42 to 71 gm. were used as experimental animals. Five male rats were put on each diet in the soybean series; and five males and five females, in the red clover series.

Phosphorus analyses of the hays used in the experimental diets were as follows: the low phosphorus soybean hay, 0.12% (0.27% P_2O_5); the high phosphorus soybean hay, 0.21% (0.48% P_2O_5); the low phosphorus red clover, 0.12% (0.27% P_2O_5); and the high phosphorus red clover, 0.31% (0.71% P_2O_5).

As indicated in tables 1 and 2, the diets were so constructed that energy, fiber, mineral, and vitamin contents were the same for all animals in a given series, insofar as was possible, and all essential nutrients were present in adequate amounts with the exception of phosphorus. The amount of phosphorus for each diet was approximately 0.16% since, as previously

TABLE 1

Composition of experimental diets

COMPONENT	SOYBEAN HAY DIET			RED CLOVER HAY DIET		
	Control	Low P hay	High P hay	Control	Low P hay	High P hay
	%	%	%	%	%	%
Hay	0	67	39	0	69	26
Lactalbumin	18	7	13	18	11	15
Starch + yeast extract	45	12	22	49	6	34
Agar	23	0	12	19	0	11
Salt mixture[1]	4	4	4	4	4	4
Butterfat	8	8	8	8	8	8
Cod liver oil	2	2	2	2	2	2

[1] Osborne and Mendel salt mixture modified to supply 0.16% phosphorus for each diet and equal amounts of calcium for each diet in a given series. Copper sulfate has also been added to the salt mixture.

TABLE 2

Distribution of foodstuffs among the various constituents of the soybean and red clover diets

DIETARY FACTOR		SOYBEAN HAY DIETS			RED CLOVER HAY DIETS		
		Control	Low P hay	High P hay	Control	Low P hay	High P hay
		%	%	%	%	%	%
Protein:	Hay	0	11	5	0	7	3
	Lactalbumin	18	7	13	18	11	15
	Total	18	18	18	18	18	18
"N-free extract":	Hay	0	22	15	0	28	10
	Starch	45	12	22	49	6	34
	Total	45	34	37	49	34	44
Fat:	Butter	8	8	8	8	8	8
	Cod liver oil	2	2	2	2	2	2
Fiber:	Hay	0	23	11	0	19	8
	Agar	23	0	12	19	0	11
	Total	23	23	23	19	19	19
Calcium:	Hay	0	0.710	0.386	0	0.959	0.263
	Lactalbumin	0.036	0.014	0.026	0.036	0.022	0.030
	$CaCO_3$	0.653	0	0.301	0.868	0	0.686
	Total	0.689	0.724	0.713	0.904	0.981	0.979
Phosphorus:	Hay	0	0.080	0.080	0	0.080	0.080
	Lactalbumin	0.027	0.011	0.020	0.027	0.017	0.023
	H_3PO_4	0.118	0.077	0.062	0.132	0.066	0.058
	Total	0.145	0.168	0.162	0.159	0.163	0.161
Ca/P ratios		4.8	4.3	4.4	5.7	6.0	6.1

shown, this furnishes about the minimal amount required for normal growth and bone development. The amount of calcium in each diet of a given series was the same, and was determined by the diet which originally contained the most. Since the red clover contained a higher percentage of calcium than the soybean hay, the Ca/P ratio in the red clover diets was higher than that in the soybean diets, namely, 5.7 : 1 and 4.8 : 1, respectively.

The triplicate feeding method employed in the previous studies with lespedeza sericea and alfalfa was used here. Of three animals of the same sex, litter, and approximately the same weight, one animal was put on the control diet, and one each on the experimental diets being compared. The food consumption of the trio was kept uniform.

The animals were started on the experimental diets at 30 to 31 days of age and were killed at 60 to 61 days of age. Rate of growth and phosphorus retentions were used as criteria in comparing the effects of the different diets on the rats. The rats were ashed at approximately 550°C. and analyzed for phosphorus by the volumetric method essentially as approved by the Association of Official Agricultural Chemists ('35).

RESULTS

Table 3 summarizes the average results obtained for all animals. These results substantiate those obtained under similar experimental conditions using lespedeza and alfalfa hays. The rats, male or female, receiving one-half their total phosphorus from a low phosphorus hay, either soybean or red clover, were conspicuously less thrifty animals than either the control animals or those deriving one-half the phosphorus from a hay high in phosphorus content whether judged on the basis of gain in weight, body length, or the amounts of phosphorus retained in the body. In the case of the soybean hay, the rats fed the control, low phosphorus hay diet, and the high phosphorus hay diet gained on the average 68, 31, and 46 gm., respectively, during the 30-day experimental period; and stored in their bodies, on the average, 0.638, 0.479,

TABLE 3

Summary of average results for experimental animals in growth and phosphorus intake and retention including summaries from variance analyses for phosphorus content of rat at 60–61 days of age

EXPERIMENTAL GROUP	NO. OF CASES	WEIGHT			LENGTH OF TRUNK	TOTAL FECES DRY WT.	FOOD CONSUMPTION WITH A.D.[1]	TOTAL P IN FOOD WITH A.D.	P IN RAT AT 60–61 DAYS WITH A.D.	P IN RAT AT 30–31 DAYS WITH A.D.	P STORED FROM FOOD WITH A.D.	P FROM FOOD
		Initial net	Final net	Gain net								
		gm.	gm.	gm.	cm.	gm.	gm.	gm.	gm.	gm.	gm.	%
Triplicate feeding												
Soybean—males												
Control	5	55	123	68	15.4	54	238 ± 16	0.345 ± 0.023	0.638 ± 0.027	0.293 ± 0.019	0.345 ± 0.020	100
Low P	5	55	86	31	14.0	114	239 ± 17	0.401 ± 0.026	0.479 ± 0.025	0.292 ± 0.012	0.186 ± 0.016	46
High P	5	56	102	46	14.6	98	239 ± 19	0.376 ± 0.036	0.540 ± 0.029	0.297 ± 0.008	0.243 ± 0.021	65
F value for diets									65.05[2]			
Significant difference									0.032			
Red clover—males												
Control	5	51	105	54	15.4	57	239 ± 24	0.379 ± 0.038	0.535 ± 0.041	0.272 ± 0.042	0.263 ± 0.024	69
Low P	5	51	63	12	13.4	121	239 ± 23	0.389 ± 0.038	0.372 ± 0.047	0.271 ± 0.031	0.101 ± 0.026	26
High P	5	52	92	40	14.6	91	241 ± 24	0.387 ± 0.039	0.492 ± 0.044	0.274 ± 0.031	0.218 ± 0.024	56
F value for diets									79.23[2]			
Significant difference									0.031			
Red clover—females												
Control	5	52	89	37	14.6	51	217 ± 19	0.345 ± 0.030	0.498 ± 0.036	0.276 ± 0.021	0.223 ± 0.032	65
Low P	5	52	58	6	13.0	113	222 ± 19	0.360 ± 0.030	0.404 ± 0.029	0.277 ± 0.016	0.127 ± 0.014	35
High P	5	52	80	28	14.2	84	222 ± 19	0.357 ± 0.031	0.483 ± 0.031	0.275 ± 0.018	0.208 ± 0.036	58
F value for diets									23.69[2]			
Significant difference									0.034			

[1] A.D. = average deviation.

[2] F value exceeds the 1% point.

and 0.540 gm. phosphorus, respectively. As shown in table 3, the control rats stored 100% of the readily available phosphorus furnished in the food. Those fed the low phosphorus hay diet retained less than 50% of the dietary phosphorus, all of which could have been obtained from the phosphorus of the salt mixture. The animals receiving the high phosphorus hay diet retained more than 50% of the food phosphorus and therefore must have obtained some of it from the hay. It is to be noted that the amounts of feces eliminated on each diet are in inverse ratio to the gains in weight and the phosphorus retained by the animals. Similar relationships hold for the rats, male and female, in the red clover series.

A variance analysis of the data gives evidence that the differences in phosphorus retention on the different diets within a given series are highly significant. The average difference in phosphorus retention of the animals on the low phosphorus hay diets and those on the high phosphorus hay diets in each series is larger than the amount required for significance.

It is of interest to point out that the gains in weight of the animals and the percentages of phosphorus of the food stored are considerably less for each corresponding diet in the red clover than in the soybean series. Considering only the males, it will be seen that it so happened that the food consumptions were nearly identical for the two series. The only obvious difference between the diets in the two series, especially in relation to the control diets, is a difference in Ca/P ratio which is more unfavorable in the red clover diets than in the soybean diets. Applying "Student's" t-test, according to Goulden ('39), to the average difference of 0.103 gm. in the amount of phosphorus at 60 days between the control rats in the red clover series and the soybean series, it is found that in only two cases in 100 would a difference of that size occur by chance.

It therefore seemed worth while to collect all the available data concerning Ca/P ratios obtained with the animals used in the previous work on lespedeza and alfalfa hays. Considering only the controls in any series of animals, which

were thus fed practically the same diet except for the amount of calcium included in the salt mixture, there seems to be a relationship between Ca/P ratio and average amount of phosphorus stored in the rat at 60 days. It is to be remembered that in all cases the amount of phosphorus fed was at a minimal level.

The data in table 4 indicate that as the Ca/P ratio increases, the food consumption and the amount of phosphorus stored in the rat decrease. These results are in agreement both with the work of Bethke, Kick and Wilder ('32) who state that increasing the Ca/P ratio from 1 to 5 causes progressive decrease in growth, bone ash, and percentage inorganic blood phosphorus in the rat; and with Brown, Shohl, Chapman,

TABLE 4

Comparison of amount of phosphorus stored on the basis of Ca/P ratios

NO. OF RATS	Ca/P RATIO	FOOD CONSUMPTION	P IN RAT AT 60 DAYS
		gm.	*gm.*
4	1.5	349 ± 17	0.886 ± 0.013
33	3.3	309 ± 40	0.700 ± 0.105
15	4.8	251 ± 36	0.650 ± 0.069
6	5.7	232 ± 25	0.521 ± 0.053

Rose and Sauerwein ('32) who find that at a given level of Ca (or P), increasing the ratio of Ca/P intensifies the degree of rickets in rats. They emphasize that both the level and the ratio are necessary adequately to characterize the ricketogenic properties of a diet. The question of Ca/P relationships is emphasized not so much in connection with the experimental work presented in this paper, since Ca/P ratios were carefully controlled in animals being compared directly, but to point out the practical implication which follows from the fact, as reported by Weathers ('38), that when hays which are low in phosphorus are compared with high phosphorus hays of the same type, they seem to have a higher Ca/P ratio which in itself may affect adversely the nutritional quality of a low phosphorus hay.

SUMMARY OF RESULTS

1. When the experimental diets contain phosphorus at a minimal level of adequacy and all other nutrients at optimal levels and essentially the same for all diets, the phosphorus of a low phosphorus soybean or red clover hay is less available to the rat for growth and bone development than the phosphorus of a high phosphorus hay of the same type.

2. Evidence has been presented indicating that for the rat, with the total phosphorus of the diet at a minimal level, an increase in Ca/P ratio is accompanied by a decrease in food consumption and in the amount of phosphorus stored in the animal's body.

CONCLUSION

The present paper extends to soybean and red clover hay the results concerning the availability of phosphorus in lespedeza sericea and alfalfa. For four different kinds of leguminous hay, it has now been shown that rats consuming a relatively large amount of a low phosphorus hay to supply the minimal phosphorus requirement grow less and store less phosphorus over a given time than rats receiving the same amount of phosphorus from a smaller amount of a high phosphorus hay of the same type. The explanation for this difference in availability of the phosphorus seems to be related to the fact that there is a marked increase in the amount of feces eliminated on the diet containing the low phosphorus hay as compared to the amount eliminated on the diet with the high phosphorus hay. The possibility is recognized that the differences in phosphorus content of the hay may accompany other differences in quality of the hay not brought out by routine analysis, such as in the fraction designated as "nitrogen-free extract."

LITERATURE CITED

ASSOCIATION OF OFFICIAL AGRICULTURAL CHEMISTS 1935 Official and tentative methods of analysis, 4th ed.

BETHKE, R. M., C. H. KICK AND W. WILDER 1932 The effect of the calcium-phosphorus relationship on growth, calcification, and blood composition of the rat. J. Biol. Chem., vol. 98, pp. 389–403.

BROWN, H. B., A. T. SHOHL, E. E. CHAPMAN, C. S. ROSE AND E. M. SAUERWEIN 1932 Rickets in rats. XIII. The effect of various levels and ratios of calcium to phosphorus in the diet upon the production of rickets. J. Biol. Chem., vol. 98, pp. 207–214.

GOULDEN, C. H. 1939 Methods of statistical analysis. John Wiley and Sons, Inc., New York, p. 41.

WEATHERS, E. K. 1938 Mineral and nitrogen content of lespedezas and other hay crops in Tennessee. Univ. of Tenn. Agric. Exp't. Sta. Bull. no. 166, 32 pp.

WILLIAMS, D. E., F. L. MacLEOD AND E. MORRELL 1940 Availability to white rats of phosphorus in lespedeza sericea and alfalfa hays. J. Nutrition, vol. 19, pp. 251–262.

THE PREVENTION AND CURE OF NUTRITIONAL MUSCULAR DYSTROPHY IN THE RABBIT BY ALPHA-TOCOPHEROL IN THE ABSENCE OF A WATER-SOLUBLE FACTOR

C. G. MACKENZIE, MILTON D. LEVINE AND E. V. McCOLLUM
Department of Biochemistry, School of Hygiene and Public Health, The Johns Hopkins University, Baltimore, Maryland

(Received for publication July 11, 1940)

Morgulis and his co-workers (Morgulis and Spencer, '36; Morgulis, Wilder and Eppstein, '38; Morgulis, '38 and '38–'39) have reported that nutritional muscular dystrophy in the rabbit is a multiple deficiency disease produced by the absence of either a water-soluble or a fat-soluble factor. We have recently identified the fat-soluble antidystrophy factor as alpha-tocopherol (Mackenzie and McCollum, '39, '40). Support for the dual nature of the deficiency may be found in the early failure of Goettsch and Pappenheimer ('31) to prevent the disease with wheat germ oil.

Confirmation of the observation of Morgulis et al. that the absence of either of two factors results in the same reaction of a specific tissue would be of great theoretical as well as practical importance. A similar interrelationship in other species has not been excluded, since the diets used to produce vitamin E deficiency have included a source of the postulated water-soluble factor, namely, yeast. Consequently the conclusion of Goettsch and Ritzmann ('39) that the rat can dispense with the water-soluble factor was not valid.

Attempts by us during the past year to demonstrate that a water-soluble factor is essential for the integrity of the skeletal muscles of rabbits fed the diet employed by Morgulis have failed. We have, however, extended our observations on the

alpha-tocopherol requirements of the rabbit, and obtained information on the production of chronic dystrophy.

METHODS

Animals and their care. The methods employed both for diagnosing dystrophy and detecting a response to a positive supplement, and for the housing and care of the animals, have been previously described (Mackenzie and McCollum, '40). Attention is called to the practice of withholding food for several hours before and after administering supplements to prevent their mixture with food in the stomach. Supplements were fed by mouth from syringes equipped with long blunt needles.

Young rabbits, weighing from 300 to 400 gm., were obtained from the colony of the Department of Immunology of the School of Hygiene and Public Health. This stock is snuffles-free and the number of deaths from coccidiosis is low. The creatine excretion of these animals is relatively constant, and does not exceed 15 mg. daily or 10 mg. per kilogram of body weight. An appreciable increase has been invariably accompanied by muscle lesions. The necessity for establishing these facts was recently demonstrated when we obtained from a dealer a group of rabbits exhibiting a high and fluctuating creatinuria.

Diets. The basal diet used by us was the one generally employed by Morgulis and his co-workers, diet 13 of Goettsch and Pappenheimer ('31). In most of our experiments this diet was modified by increasing the U.S.P. hydrated ferric chloride from a level of 1% to 1.67%. Consequently the level of anhydrous ferric chloride was 1%. The modified diet 13 has been designated as no. 133.

Supplements. Petroleum ether-extracted wheat germ was prepared by extracting fresh wheat germ [1] in the Soxhlet

[1] Wheat germ was obtained from the Russell Miller Milling Company, Minneapolis, Minnesota.

apparatus or in a continuous extractor with C. P. petroleum ether (B. P. 35 to 60°C.) for 24 hours. Ethyl ether-extracted wheat germ was prepared by extracting wheat germ in the continuous extractor with cool U.S.P. ethyl ether for 24 hours.

In preparing a concentrate of the water-soluble factor the procedure of Morgulis was closely followed. Petroleum ether-extracted wheat germ was covered with 70% alcohol, stirred vigorously for 2 hours and allowed to stand overnight at room temperature. The extract was filtered off and the extraction repeated until the filtrate was colorless. The filtrates were combined and the alcohol removed by distillation under reduced pressure from a water bath at 40°C. The precipitate formed was filtered off and discarded. Benzoic acid was added to the filtrate as a preservative. Extracts were prepared fresh weekly and stored in the refrigerator in a glass stoppered flask.

An alternative procedure was based on the extractability of the water-soluble factor by acetone but not by ether, as reported by Morgulis, Wilder and Eppstein ('38). Ethyl ether-extracted wheat germ was extracted with warm C. P. acetone in the Soxhlet apparatus for 24 hours. During this time a white precipitate was formed in the extraction flasks. The acetone was decanted and distilled off at the water pump. The residue was taken up in water as was the precipitate in the extraction flasks and the two combined. Benzoic acid was added as a preservative.

The petroleum ether extract of wheat germ was used as a source of wheat germ oil. The petroleum ether solution was filtered and the solvent removed at the water pump. The oil was stored in the refrigerator.

Synthetic alpha-tocopherol [2] was used as the source of vitamin E. Ethyl laurate solutions containing 20 and 50 mg. per cubic centimeter were prepared at least every 2 weeks and kept in glass stoppered bottles in the refrigerator.

[2] We are indebted to Merck and Company, Inc., for the supply of alpha-tocopherol.

EXPERIMENTAL

Increasing the ferric chloride [3] content of diet 13 from 10 to 16.7 gm. per kilogram resulted in the earlier death of young animals fed the unsupplemented diet as shown in table 1. The symptoms of dystrophy were frequently attended and sometimes obscured by diarrhea and respiratory infections. These complications considerably shortened the survival time of many of the sick animals. While the more drastic action of diet 133 (diet 13 with increased ferric chloride) was probably due in part to the increased destruction of vitamin E, other modes of action of the salt, for example on mineral metabolism, cannot be eliminated. As will be shown later, however, such

TABLE 1

The effect of increasing the ferric chloride [1] content of diet 13 on the survival time of young rabbits

NUMBER OF RABBITS	AVERAGE WEIGHT	DIET	FERRIC CHLORIDE	PER CENT DEAD				
				1 week	2 weeks	3 weeks	4 weeks	5 weeks
	gm.		*per cent*					
8	380	13	1.0	0	0	0	38	50
12	390	133	1.67	0	0	34	75	84

[1] The hydrated salt is referred to.

effects must have been entirely dependent on the development of vitamin E deficiency.

The addition of alpha-tocopherol to diet 133. Sixteen young rabbits were placed on diet 133 and fed alpha-tocopherol at levels of 3 mg., 1 mg., and 0.5 mg. daily, 6 days a week. Five more animals were fed the diet without alpha-tocopherol. Administration of the supplement and care of the animals have been described in the section entitled Methods. The diagnosis of dystrophy was based either on an increased excretion of creatine or the presence of lesions in the thigh muscles.

It is apparent from table 2 that the 3 mg. level of the vitamin afforded complete protection against muscular dystro-

[3] Here and throughout the remainder of the paper ferric chloride refers to the hydrated salt.

TABLE 2

The preventive action of alpha-tocopherol fed to rabbits reared and maintained on dystrophy-producing diet 133

ANIMAL	SEX	DAILY DOSE OF α-t [1]	INITIAL WEIGHT	TIME TO ABNORMAL CREATINURIA	MAXIMUM WEIGHT	LOSS IN WEIGHT	DURATION OF EXPERIMENT	FIRST PHYSICAL SYMPTOMS	MICROSCOPIC LESIONS [3]	COMMENTS
		mg.	*gm.*	*weeks* [2]	*gm.*	*gm.*	*weeks*	*weeks*		
52	♀	3	350		1750	0	12		.....	Killed
46	♀	3	290		1730	0	12		.....	Killed
55	♂	3	480		2380	0	14		.....	Killed
53	♂	3	440		2550	0	17		.....	Killed
66H	♀	3	850	..	2880	0	20		.....	Killed
67H	♂	5	950		3570	0	21		.....	Killed
50	♂	3	400		2540	0	24		.....	Killed
22B	♂	1	470	12	2310	0	18		± [4]	Killed
22D	♀	1	290	12	1860	0	17		+	Killed
25	♀	1	300		2680	120	31		+±	Killed
22C	♀	0.5	480		1610	60	8	8	+++	Died. Diarrhea
71	♀	0.5	330	7	1710	50	9	8	++	Killed
74	♂	0.5	370	7	1900	0	12			Discontinued
62		0.5	300	6	1760	100	12		++	Died. Diarrhea
22A	♂	0.5	500	9	2490	0	16			Discontinued
72	♂	0.5	420	<11	3170	0	20		+±	Killed
70	♂	0	320	3	980	220	3½	3½	++++	Died. Respiratory infection
73		0	305	3	850	170	4	3½	++++	Killed. Diarrhea
77	♀	0	430	2½	940	110	3	3	++++	Killed
79	♂	0	400	3	970	200	4	3	++++	Died. Diarrhea
81		0	430	2	950	180	3	2½	+++	Killed. Respiratory infection

[1] Alpha-tocopherol given 6 days a week.

[2] The week during which the daily creatine excretion reached 20 mg. has been selected as the beginning of definitely abnormal creatinuria.

[3] Biceps femoris and vastus lateralis examined.

[4] Only one or two lesions (accumulations of nuclei) per section. Creatine excretion at 16 weeks had fallen to 4 to 5 mg. per kilogram of body weight.

phy. The 1 mg. level of alpha-tocopherol did not suffice to prevent symptoms of dystrophy indefinitely, though in the case of rabbit 22 B the slightly abnormal creatinuria occurring at the twelfth week was transitory, declining from 4 to 5 mg. per kilogram of body weight at the sixteenth to eighteenth week. At the eighteenth week the microscopic lesions of the muscles were confined to one or two masses of nuclei per section. In the case of the other two animals the lesions were more extensive. However, rabbit no. 25 was carried for 31 weeks. None of these animals exhibited physical symptoms.

Considerable variation was observed in the rabbits fed 0.5 mg. of alpha-tocopherol, as shown in table 2. One animal died at 8 weeks and one at 12 weeks (both had diarrhea), while two others were alive at 16 and 20 weeks. Rabbits 22 C and 71 were the only ones to show physical symptoms of dystrophy during the experiment. The 0.5 mg. level of alpha-tocopherol postponed the rise of urinary creatine from 3 weeks (2.0 to 3.5 for unsupplemented animals) to 7 (6 to 9) weeks. This in turn is earlier than the time of creatine rise found for two of the rabbits on the 1.0 mg. level.

From this experiment it was concluded that the administration of sufficiently large amounts of alpha-tocopherol to rabbits on diet 133 prevented dystrophy. The possibility that we were working with a special strain of animals that did not require the postulated water-soluble factor of Morgulis, and that this factor was essential for other rabbits, was partially eliminated by including in the experiment two rabbits, 66H and 67H, that were unrelated to our main stock. We next turned our attention to the question of a quantitative relationship between alpha-tocopherol and a water-soluble factor.

The effect of defatted wheat germ and its extracts on chronic dystrophy. Chronic dystrophy was produced in four rabbits on diet 133 through the administration of subminimal doses of alpha-tocopherol. The defatted wheat germ supplements were mixed in the basal diet daily at a 10% level. Higher levels resulted in reduced and incomplete food consumption. Extracts of defatted germ were given by mouth as described

in the section headed Methods. The results of this experiment as shown by table 3 were negative. The ability of the animals to respond to positive supplements was confirmed by feeding alpha-tocopherol or wheat germ oil at the end of the experiment.

TABLE 3

The effect of defatted wheat germ and its extracts on the creatinuria of rabbits with chronic dystrophy on diet 133 plus subminimal levels of alpha-tocopherol

ANIMAL	SEX	ALPHA-TOCOPHEROL DAILY [1]	DAYS ON DIET	DAILY SUPPLEMENTS	DAYS OF TREATMENT	AVERAGE WEIGHTS, GM.	AVERAGE DAILY CREATININE, MG. PER KGM. OF WEIGHT [2]	AVERAGE DAILY CREATINE, MG. PER KGM. OF WEIGHT [2]
		mg.						
102	♂	0.5	69	E.E.W.G. = 6 gm. Acet. E ⌓ 20 gm.	10	2130	28	10
		0.5	82		8	2320	30	19
		0.5	90	Al. E ⌓ 20 gm.	8	2420	31	15
		0.5	98	P.E.W.G. = 6 gm. Al. E ⌓ 14 gm.	14	2450	27	18
		0	113	Wheat germ oil = 0.7 cc.	14	2500	23	4
106	♀	0.5	35		15	1270	23	13
		0.5	50		13	1470	21	20
		0.5	63	Acet. E ⌓ 20 gm.	11	1620	26	20
		0.5	74	P.E.W.G. = 5 gm. Al. E ⌓ 20 gm.	9	1700	19	18
104	♂	0.5	52		11	1710	30	18
		0.5	63	P.E.W.G. = 6 gm. Al. E ⌓ 20 gm.	14	1890	26	20
		0.5	77		7	1900	25	17
		0	84	Alpha-tocopherol = 3 mg.	12	2160	26	4
105	♀	1.0	82		10	1630	37	12
		1.0	92		8	1710	36	15
		1.0	100	Al. E ⌓ 20 gm.	8	1800	32	16
		1.0	108	P.E.W.G. = 5 gm. Al. E ⌓ 20 gm.	10	1820	30	19
		0	119	Alpha-tocopherol = 3 mg.	13	1860	27	5

E.E.W.G. = Ethyl ether-extracted wheat germ.

P.E.W.G. = Petroleum ether-extracted wheat germ.

Acet. E. = Acetone extract of ethyl ether-extracted wheat germ.

Al. E. = 70% alcohol extract of petroleum ether-extracted wheat germ.

[1] Alpha-tocopherol given 6 days a week.

[2] Creatine and creatinine determinations made on 1 or 2 day collections of urine.

The response elicited by vitamin E was not dependent on the previous administration of sources of the postulated water-soluble factor. This was demonstrated by treating three animals that developed chronic dystrophy on diet 133 (in the absence of a source of the water-soluble factor) with 3 mg. of alpha-tocopherol daily. In each case the abnormal creatinuria fell rapidly to the normal level.

This experiment failed to disclose a quantitative relationship between vitamin E and a water-soluble factor, and demonstrated the cure of chronic dystrophy with vitamin E alone. Nevertheless a third possibility existed, namely, that alpha-tocopherol alone would not suffice to cure acute dystrophy because of the development of some secondary deficiency induced by low food consumption, digestive disturbances or extensive muscle damage.

The effect of alpha-tocopherol on acute dystrophy. Since, as was previously mentioned, many of the animals on unsupplemented diet 133 developed diarrhea and respiratory infections and died soon after the first signs of illness, we reverted to diet 13 in this experiment. This also enabled us to compare the results obtained with those reported in our first paper (Mackenzie and McCollum, '40) on the curative action of alpha-tocopherol administered to animals on diet 13 plus defatted wheat germ.

Seven young rabbits were placed on diet 13 and two on diet 13 plus 10% of ethyl-ether-extracted wheat germ. The average weight for the group was 330 gm. The daily creatine excretion reached 20 mg. in 21 (16 to 27) days. At the time of treatment three of the animals had stage II dystrophy and six stage III dystrophy (Mackenzie and McCollum, '40). The creatine excretion had reached an average level of 55 (40 to 80) mg. per day, or an average of 62 mg. per kilogram of body weight. Four of the animals had ceased eating. The administration of 3 mg. of alpha-tocopherol daily for 5 days resulted in a prompt and typical response in all cases. The creatine excretion dropped appreciably in from 1 to 3 days

and soon returned to the normal level. The physical symptoms disappeared completely and growth was resumed.

Four of the cured animals were continued on diet 13 without vitamin E. The daily creatine excretion once more rose to 20 mg. in from 23 to 31 days after the first dose of alpha-tocopherol. The use of smaller animals in this experiment probably accounts in part for the longer period required for the rise in creatine as contrasted with our first report of 15 to 20 days (Mackenzie and McCollum, '40). Three of the animals continued on diet 13 were allowed to develop acute dystrophy and were again cured with alpha-tocopherol.

The results reported in this section demonstrated that in our hands, and with our method of administering supplements, acute dystrophy produced on diet 13 could be consistently and repeatedly cured with alpha-tocopherol alone. It still remained for us to obtain evidence that our diets were not contaminated with the water-soluble factor reported by Morgulis and his co-workers.

The effect of incorporating wheat germ oil in diet 133. Morgulis ('38–'39) in his most recent publication states that in preventive treatments the supplements are incorporated in the diet and that in curative treatments the supplements are given to prostrated animals by pipette, but that as soon as the animals are on their feet and able to eat, the supplements are incorporated in the diet as in the case of less severely dystrophic animals. In other words, if wheat germ oil is added to the diet as a source of the fat-soluble factor the rabbits should develop dystrophy as a consequence of the water-soluble factor deficiency unless this factor is also present in the diet. In preventive experiments this is the criterion for the existence of the water-soluble factor.

Wheat germ oil prepared from a petroleum ether extract of wheat germ was mixed in diet 133 in amounts sufficient to insure the consumption of approximately 2 cc. of oil per day. This mixture was prepared in amounts sufficient to last for 3 or 4 days following the procedure of Morgulis ('38, p. 80). The oil was potent at a level of 1 cc. per day when administered

to rabbits rendered dystrophic on diet 133 plus 10% of defatted wheat germ.

The result of mixing wheat germ oil in diet 133 is shown in table 4. The first sign of dystrophy, a rise in the creatine excretion, was observed in from 35 to 40 days, and gross symptoms occurred soon thereafter. This confirmation of the observation of Morgulis ('38, '38–'39) indicated that our basal diet was deficient in the suggested water-soluble factor.

TABLE 4

Effect of the addition of wheat germ oil to diet 133. Animals 80 and 83 received in addition 10% of defatted wheat germ in the diet from the thirty-seventh to seventy-seventh day

Animal	75	76[1]	78	84	80	83
Sex	♀	♀		♂	♂	♂
Wheat germ oil daily, cc.	2.0	1.7	1.9	1.8	1.9	2.0
Initial weight, gm.	300	320	360	360	370	420
Time to abnormal creatinuria,[2] days	40	35	35	39		
Maximum creatine,[3] mg./kgm.	50	66	80	50	8	10
Maximum weight, gm.	1610	1330	1350	1170	2060	2060
Loss in weight, gm.	160	220	230	200		
Duration of experiment, days	62	40	38	38	98	98
First physical symptoms, days	53	38	35	39		

[1] Animal 76 was cured of dystrophy at the end of the experiment by omitting the oil from the diet and administering 1 cc. per day by mouth.

[2] The day on which the creatine excretion reached 20 mg. has been selected as the beginning of definitely abnormal creatinuria.

[3] Expressed as milligrams per kilogram of body weight per day.

Two additional animals on diet 133 plus wheat germ oil, that showed no signs of dystrophy on the thirty-seventh day of the experiment, were supplemented with 10% of petroleum ether-extracted wheat germ at that time (see table 4). The defatted germ was mixed in the diet and the oil then added as described above. By the seventy-seventh day these animals had shown no signs of dystrophy and the germ and oil were both discontinued. The animals were continued for 3 weeks on diet 133 at which time the creatine excretion for no. 80 and no. 83 was 3 and 6 mg. per kilogram of body weight respectively. These results suggest that the defatted wheat germ

protected the vitamin E in the oil from destruction by the diet.

Vitamin B_6. Although all of our attempts to demonstrate the existence of a water-soluble antidystrophy factor had failed we were prompted by the report of Antopol and Schotland ('40) on the beneficial results obtained with vitamin B_6 in cases of human pseudo-hypertrophic muscular dystrophy to try the effect of the vitamin on rabbits. Three rabbits on diet 13 were given 5 mg. of vitamin B_6 hydrochloride [4] daily by mouth at about 10 days before a rise in urinary creatine was to be expected. In each case severe dystrophy developed. An additional animal with a high creatinuria received a total of 400 mg. of vitamin B_6 subcutaneously over a 12-day period. The creatine remained elevated and the rabbit developed severe physical symptoms of dystrophy during the treatment. While this experiment did not eliminate a transitory effect of B_6 on the dystrophic rabbit muscle, it showed that B_6 in large amounts cannot replace vitamin E in preventing or curing the disease.

DISCUSSION

We have attempted to confirm the observation of Morgulis and co-workers (Morgulis and Spencer, '36; Morgulis, Wilder and Eppstein, '38; Morgulis, '38 and '38–'39) that rabbits reared on diet 13 of Goettsch and Pappenheimer ('31) require for the structural integrity of their skeletal muscles two vitamins, the absence of either of which results in the necrosis of these muscles. Our experiments indicate that, when proper precautions are taken to prevent the destruction of vitamin E by the diet, the dystrophy may be prevented or cured by alpha-tocopherol alone. Furthermore, we have been unable to demonstrate a quantitative or synergistic relationship between alpha-tocopherol and a water-soluble factor in the cure of chronic dystrophy produced by feeding subminimal doses of alpha-tocopherol.

[4] We are indebted to Merck and Company, Inc., for the supply of vitamin B_6 hydrochloride.

The fact that the addition of a potent wheat germ oil to the dystrophy-producing diet failed to prevent the disease indicates that our diet was, according to the criteria employed by Morgulis, deficient in the postulated water-soluble factor. A plausible explanation for the discrepancies between our results and those obtained by Morgulis is furnished by the hypothesis that defatted wheat germ contains one or more substances protecting vitamin E from destruction by the rancid diet. Some evidence that this may be the case has been presented. This question is being made the subject of further investigation.

The failure of Goettsch and Pappenheimer ('31) to prevent dystrophy in rabbits even though wheat germ oil was administered by mouth was probably due to the fact that their animals had continual access to the diet. We have observed (unpublished data) that rabbits having continual access to an E-deficient diet and receiving ample amounts of alpha-tocopherol by mouth become dystrophic when cod liver oil is added to the diet.

We have fully confirmed our earlier observations (Mackenzie and McCollum, '40) on the prevention and cure of nutritional muscular dystrophy in the rabbit with alpha-tocopherol. The level of the vitamin necessary to prevent muscle lesions in growing rabbits lies between 6 and 18 mg. per week though 3 mg. per week will greatly prolong life. On the latter dosage animals may grow for months without exhibiting physical symptoms. However, there is a definite creatinuria, and microscopic lesions are found at autopsy. Taken in conjunction with the duration of the effect of the 15 mg. curative dose, the daily requirement of the rabbit for alpha-tocopherol may be again roughly fixed at 0.6 to 1.0 mg. per kilogram of body weight. An appreciable gap exists between this figure, representing the amount necessary to prevent muscle lesions, and the intake that will suffice to maintain normal growth and prevent gross symptoms.

It thus appears that a number of muscle fibers can be damaged without seriously affecting the health of the animal

(though its activity is possibly reduced) and that the number of fibers damaged is inversely proportional to the vitamin E intake. The data in tables 2 and 3 indicate that the quantitative aspect of such a relationship varies between individuals, possibly as a result of differences in the absorption and transportation of the vitamin and the degree of muscular activity.

The relative constancy of the creatinuria from week to week in cases of chronic dystrophy suggests that this relationship is maintained by an equilibrium between degenerative and regenerative processes. Histological examinations of the muscles of animals with chronic dystrophy tend to confirm this hypothesis. All of these considerations support the theory previously advanced (Mackenzie and McCollum, '40) that vitamin E is directly involved in the metabolism of the rabbit's voluntary muscles. The comparative study of the distribution of vitamin E in the tissues of several species and in muscles showing different degrees of injury is planned.

The production of chronic dystrophy through the administration of from 3 to 6 mg. of alpha-tocopherol per week furnishes excellent material for studying the metabolism of the dystrophic animal in the absence of complications arising from loss of weight, reduced food consumption or secondary pathological conditions.

SUMMARY

1. We have been unable to confirm the report of Morgulis and co-workers that rabbits fed diet 13 require two factors for the structural integrity of their voluntary muscles. The muscular dystrophy produced on this diet has been prevented or cured by alpha-tocopherol without the addition of a water-soluble factor.

2. A quantitative relationship between alpha-tocopherol and a water-soluble factor could not be demonstrated.

3. Preventive and curative experiments place the average daily antidystrophy requirement of the rabbit for alpha-tocopherol between 0.6 and 1.0 mg. per kilogram of body weight.

4. The production of chronic dystrophy has been described and the symptoms discussed with reference to the action of vitamin E in the rabbit.

LITERATURE CITED

ANTOPOL, W., AND C. E. SCHOTLAND 1940 The use of vitamin B_6 in pseudo-hypertrophic muscular dystrophy. J. Am. Med. Assoc., vol. 114, p. 1058.

GOETTSCH, M., AND A. M. PAPPENHEIMER 1931 Nutritional muscular dystrophy in the guinea pig and rabbit. J. Exp. Med., vol. 54, p. 145.

GOETTSCH, M., AND J. RITZMANN 1939 The preventive effect of wheat germ oils and of α-tocopherol in nutritional muscular dystrophy of young rats. J. Nutrition, vol. 17, p. 371.

MACKENZIE, C. G., AND E. V MCCOLLUM 1939 Vitamin E and nutritional muscular dystrophy. Science, vol. 89, p. 370.

———— 1940 The cure of nutritional muscular dystrophy in the rabbit by alpha-tocopherol and its effect on creatine metabolism. J. Nutrition, vol. 19, p. 345.

MORGULIS, S. 1938 Nutritional Muscle Dystrophy. Hermann and Cie, Paris.

———— 1938–1939 Nutritional muscular dystrophy. Zeit. f. Vitaminforschung, vol. 8, p. 220.

MORGULIS, S., AND H. C. SPENCER 1936 A study of the dietary factors concerned in nutritional muscular dystrophy. J. Nutrition, vol. 11, p. 573.

MORGULIS, S., V. M., WILDER AND S. H. EPPSTEIN 1938 Further studies on dietary factors associated with nutritional muscular dystrophy. J. Nutrition, vol. 16, p. 219.